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14. ABSTRACT

In this proposal we show that ligation of IL-17 to IL-17R plays a critical role in rheumatoid arthritis (RA) joint vascularization. However, less is known about the pro-angiogenic factors downstream of IL-17 cascade that could indirectly contribute to RA angiogenesis. In this progress report, we discovered a novel chemokine (CCL28) and its corresponding receptor (CCR10) that is modulated by IL-17 in RA myeloid cells. We uncover for the first time the expression pattern, regulation and function of this IL-17 downstream pathway. We further provide strong evidence that supports utilizing the CCL28/CCR10 cascade as a potential therapeutic target in RA. These findings were recently published in the highest ranked rheumatology journal (Annals of Rheumatic Disease) and the discoveries were highlighted in 11 other scientific journals.

15. SUBJECT TERMS

IL-7, Angiogenesis, Rheumatoid Arthritis, experimental arthritis, VEGF, CXCL1, CXCL5, CCL28, and CCR10

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The specific aims of this proposal have remained unchanged and are performed as initially proposed.

INTRODUCTION

TH-17 cells are a newly discovered CD4 helper T-cells that produce interleukin-17A (also known as IL-17). IL-17 is found in Rheumatoid Arthritis (RA) synovial tissue and fluid, and the percentage of TH-17 cells is significantly higher in RA synovial fluid compared to RA or normal peripheral blood (1, 2). IL-17 has been shown to have a profound effect in experimental models of arthritis however its role in Rheumatoid Arthritis is undefined. Angiogenesis is an early and a critical event in the pathogenesis of RA. Since our preliminary data suggests that IL-17 plays an important role in RA angiogenesis, this grant was proposed to determine the mechanism by which IL-17 induces neovascularization.

Our overriding hypothesis is that IL-17 mediates angiogenesis in RA through activation of the PI3K pathway, and this effect may be dependent or independent of other proangiogenic factors. To test this hypothesis we will investigate the contribution of IL-17 and its receptors to RA synovial fluid-mediated endothelial migration and blood vessel growth. We will also identify signaling pathways that are involved in IL-17-mediated neovascularization and examine whether mice deficient in a particular signaling pathway are unable to mediate neovascularization through IL-17. To further examine the role of IL-17 and its synergistic effect with other factors in inducing HMVEC migration *in vivo*. Additionally, the indirect role of IL-17 in induction of angiogenesis and arthritis will be examined by blocking the effect of relevant IL-17 associated pro-angiogenic factors in *in vitro* and *in vivo* models of angiogenesis and experimental arthritis. The unmet need is to elucidate the mechanisms by which IL-17 mediates angiogenesis in RA and to determine whether targeting IL-17 and/or its intermediary molecules will provide a therapeutic intervention for RA patients.

KEY WORDS

Rheumatoid arthritis, angiogenesis, IL-17, VEGF, CXCL1, CXCL5, endothelial cells

OVERALL POJECT SUMMARY

In **task 1**, we proposed to examine the mechanism by which IL-17 induces angiogenesis, (time frame, months 1-13).

A summary of the 2011 progress report: In our progress report submitted in July of 2011 we addressed the specific aims proposed in Task 1 by demonstrating that ligation of IL-17 to IL-17RC but not IL-17 RA is responsible for the direct effect of IL-17 on angiogenesis. We further documented that ligation of IL-17 to IL-17RC mediates endothelial migration and tube formation via activation of AKT1/PI3K pathway. IL-17 expressed in the joint synovial fluid plays a crucial role in RA pathogenesis since neutralization of IL-17 in the fluid or IL-17RC on endothelial cells markedly reduces endothelial homing and vessel formation. *In vivo* we demonstrate for the first time that local expression of IL-17 induces joint inflammation that is associated with increased vascularization. Confirming these observations we also show that hemoglobin concentration is 10 fold higher in IL-17 containing Matigel plugs compared to PBS controls. These results validate that IL-17 promote RA angiogenesis directly through IL-17RC ligation and activation of AKT1 pathway. These results are published in J Immunol 2010, 184:3233-3241. Most recently others have shown that IL-17 is capable of inducing endothelial cell invasion by activating production of CXCL1/GROα and MCP-1 from RA synovial tissue fibroblasts suggesting that there may be other mechanisms involved in IL-17 mediated

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vascularization. Therefore we next studied the indirect role of IL-17 in RA pathology and angiogenesis proposed in aim 3.

TASK 2. To determine whether IL-17 plays a role in endothelial cell homing to RA ST. We will: **(time frame, months 14-21).**

2A and 2B. Examine whether IL-17 plays a role in RA ST mediated endothelial cell migration, (time frame, months 14-18).

A summary of the 2014 progress report: We show that ligation of IL-17 to IL-17R plays a critical role in rheumatoid arthritis (RA) joint vascularization. However, less is known about the pro-angiogenic factors downstream of IL-17 cascade that could indirectly contribute to RA angiogenesis. In 2014 progress report, we discovered a novel chemokine (CCL28) and its corresponding receptor (CCR10) that is modulated by IL-17 in RA myeloid cells. We uncover for the first time the expression pattern, regulation and function of this IL-17 downstream pathway. We further provide strong evidence that supports utilizing the CCL28/CCR10 cascade as a potential therapeutic target in RA. These findings were recently published in the highest ranked rheumatology journal (Annals of Rheumatic Disease) and the discoveries were highlighted in 11 other scientific journals.

2c. Determine whether IL-17 is interconnected with TNF-α in mediating angiogenesis (time frame, months 21-24). We found that ligation of toll like receptor (TLR)5 interconnects the production of TNF-α from myeloid cells with the secretion of IL-17 from TH-17 cells. Interestingly binding of TLR5 to its agonist flagellin strongly promotes TH-17 cell polarization and TLR5 mediated RA angiogenesis is in part due to IL-17 produced from TH-17 cells. Below we show evidence that the mechanism by which TLR5 exacerbates collagen induced arthritis (CIA) is driven through TH-17 cell differentiation and joint vascularization.

Post onset treatment of CIA with TLR5 agonist exacerbates joint pathology.
To further examine the mechanism by which TLR5 ligation mediates disease we first determined if TLR5 was expressed in CIA ankle joints and we later asked whether TLR5 ligation affects CIA pathology. We found that like in RA, TLR5 expression was significantly elevated in CIA lining (70%) and sublining macrophages (50%) and endothelial cells (60%) compared to control ankles (Figs. 1A-B). Further we document that when CIA mice were therapeutically treated with flagellin, joint inflammation was greatly increased in the flagellin group while the control ankle circumference remained at a plateau phase (Fig. 1C). Consistent with the clinical data, histological studies demonstrate that CIA lining thickness (30%), inflammation (40%) and bone erosion (35%) was significantly elevated by TLR5 ligation compared to control treatment (Fig. 1D-E). Collectively these results indicate that ligation of TLR5 promotes CIA disease progression. Next experiments were performed to examine whether TLR5 ligation could contribute to neovasculaization in murine models of RA. In agreement with our *in vitro* finding we show that VWF staining (40%) and hemoglobin levels (2 fold) were higher in flagellin group compared to control treated CIA mice (Figs. 1F-H). These results suggest that both in RA and CIA elevated joint vascularization is a mechanism by which TLR5 mediates joint pathology.
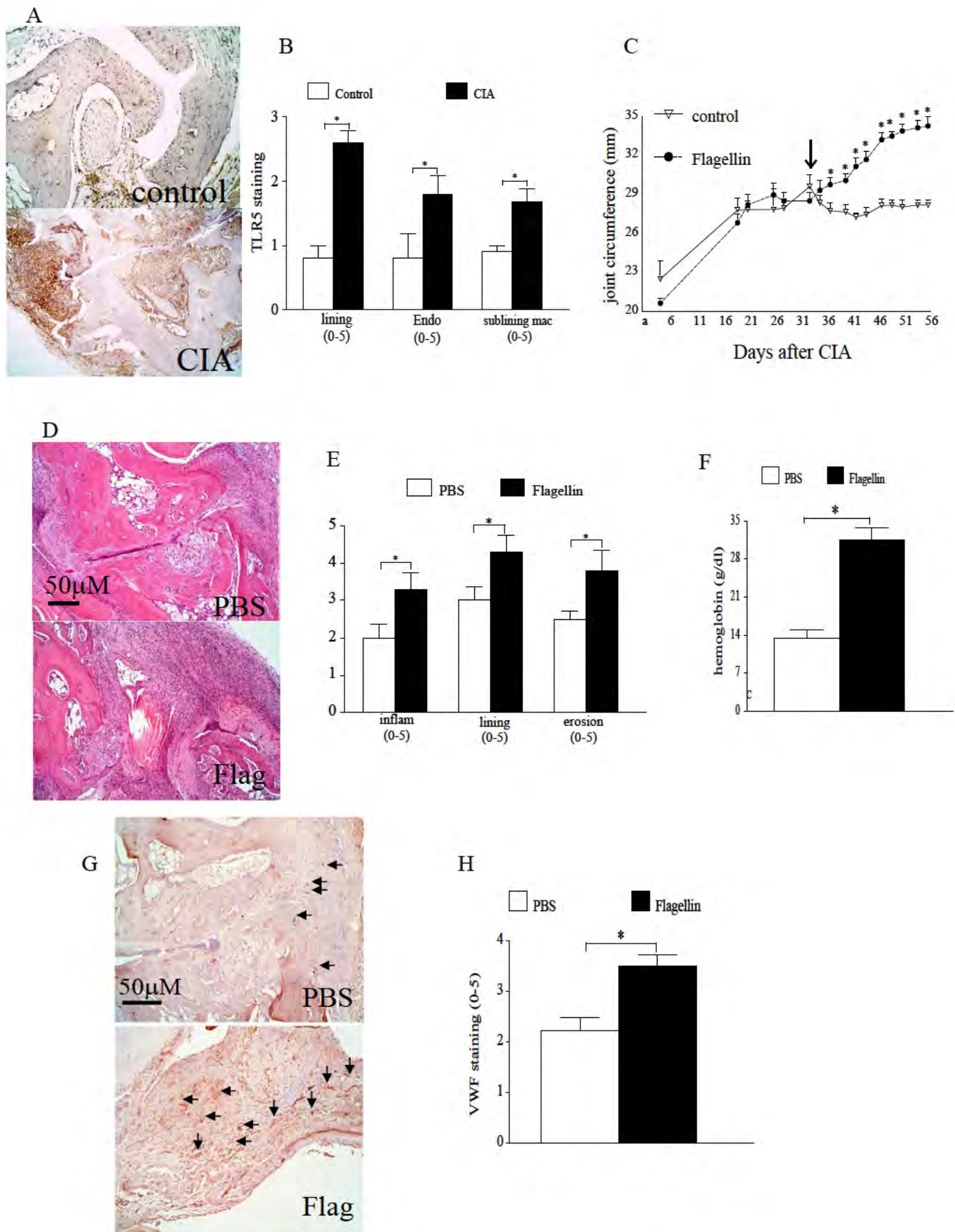
Figure 1. CIA disease severity and vascularization is markedly enhanced by post onset treatment of TLR5 agonist. A. Ankles harvested from PBS control or CIA induced mice were stained with anti-TLR5 antibody. **B.** TLR5 positive immunostaining was scored on a 0-5 scale on synovial tissue lining, sublining macrophages (mac) and endothelial cells (endo) (n=5-7) (original magnification x100). **C.** Changes in joint circumference was recorded in CIA mice that were treated with PBS or flagellin (20 μg) i.p., n=10 mice. **D.** Demonstrates the representative H&E staining in control (PBS) and flagellin (Flag) treated CIA mice (original magnification x

100). **E.** Effect of flagellin post onset treatment on CIA ankle joint inflammation, lining thickness, and bone erosion was scored on a 0-5 scale, n=6-8. **F.** Hemoglobin levels are quantified in CIA ankles harvested from control and flagellin treatment groups on day 57 and results are demonstrated as hemoglobin (g/dl)/ joint weight (mg/ml). **G.** CIA synovial tissue treated with control (PBS) or flagellin (Flag) was harvested on day 57 post onset and was immunostained with anti-VWF antibody (original magnification x 100) and (**H**) endothelial staining was quantified on a 0-5 scale, n=6-9. VWF+ staining in the CIA synovial tissues is demonstrated by arrows. Values are the mean ± SE. * represents p <0.05.

TLR5 ligation promotes CIA TH-17 cell polarization.

Since CIA disease severity was enhanced by flagellin treatment, effect of the therapy was investigated on joint proinflammatory factors. Our results demonstrate that joint IL-17 protein levels were 2.5 fold greater in the flagellin treated animals compared to the control group (Fig. 2A). Based on these observations we asked whether TH-17 cell profile was differentially modulated by flagellin treatment in CIA splenocytes. Consistent with findings in CIA joint, we document that the control splenocytes had 3 fold reduced frequency of TH-17 cells compared to the flagellin treated mice (Figs. 2B). Interestingly, we show that TH-17 promoting cytokines IL-6 and IL-1β concentrations were significantly elevated (60-50% respectively) in the flagellin group compared to the PBS treated CIA ankles (Figs. 2C-D). Additionally levels of CCL20 and VEGF, two TH-17 associated factors, were elevated in the flagellin group compared to the control CIA mice (Figs. 2E-F). These results indicate that flagellin treatment promotes CIA TH-17 cell polarization through induction of joint IL-6 and IL-1β.
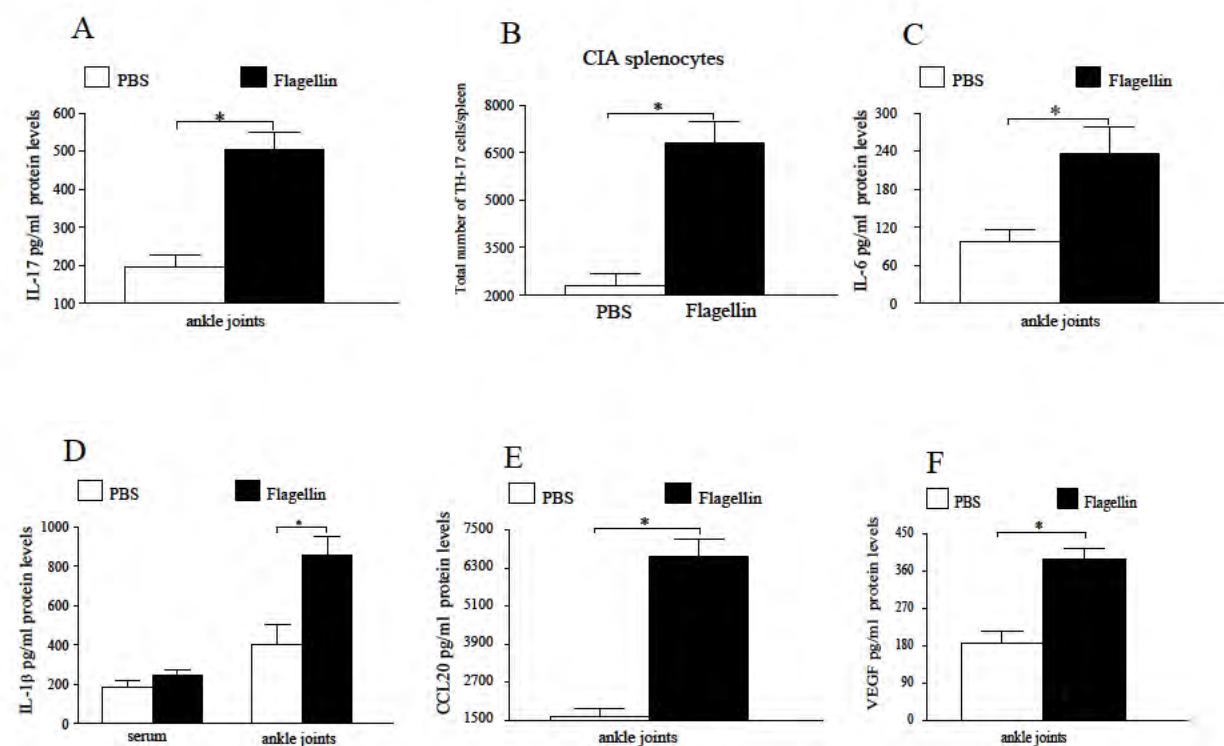

Figure 2. Polarization of TH-17 cells is elevated in flagellin compared to control treatment in CIA mice. Changes in joint IL-17 (**A**), IL-6 (**C**), IL-1β (**D**), CCL20 (**E**), VEGF (**F**) protein levels in CIA mice treated with control or flagellin were quantified by ELISA, n=6-8. **B.** Total number of TH-17 positive cells per spleen was determined by Flow cytometry analysis in CIA mice treated with control or flagellin, n=5. Values are mean ± SE. * indicates p<0.05.

Differentiation of TH-17 cells is also promoted by flagellin in normal and RA peripheral blood mononuclear cells.

We asked if ligation of TLR5 could modulate human TH-17 cell differentiation employing normal and/or RA peripheral blood mononuclear cells or whether this process was specific to mouse cells and/or

the inflammatory milieu in CIA. We found that in normal peripheral blood mononuclear cells ligation of TLR5 is capable of polarizing TH-17 cells (Figs. 3A-B) and producing elevated levels of IL-17 as well as IL-6 and IL-1β (Figs. 3C-D) in culture supernatants. Similar to normal donors, TLR5 ligation drives TH-17 cell development in RA peripheral blood mononuclear cells (Fig. 3E). Next we asked whether in RA, myeloid cell to cell contact with CD4+ cells was essential for TH-17 differentiation. We show that flagellin treated RA CD4+ T cells alone were unable to differentiate to TH-17 cells and required RA myeloid cell interaction for this process (Fig. 3F). To demonstrate that myeloid cell dependency was due to production of IL-6 and IL-1β from myeloid cells, peripheral blood mononuclear cells were pretreated with antibodies to IL-6 and IL-1β prior to TLR5 ligation. Our results clearly demonstrate that myeloid cell production of IL-6 and IL-1β is critical for flagellin mediated TH-17 polarization (Fig. 3G). Collectively these results suggest that ligation of TLR5 contributes to myeloid cell production of TH-17 promoting cytokines and TH-17 cell differentiation in RA and experimental arthritis.
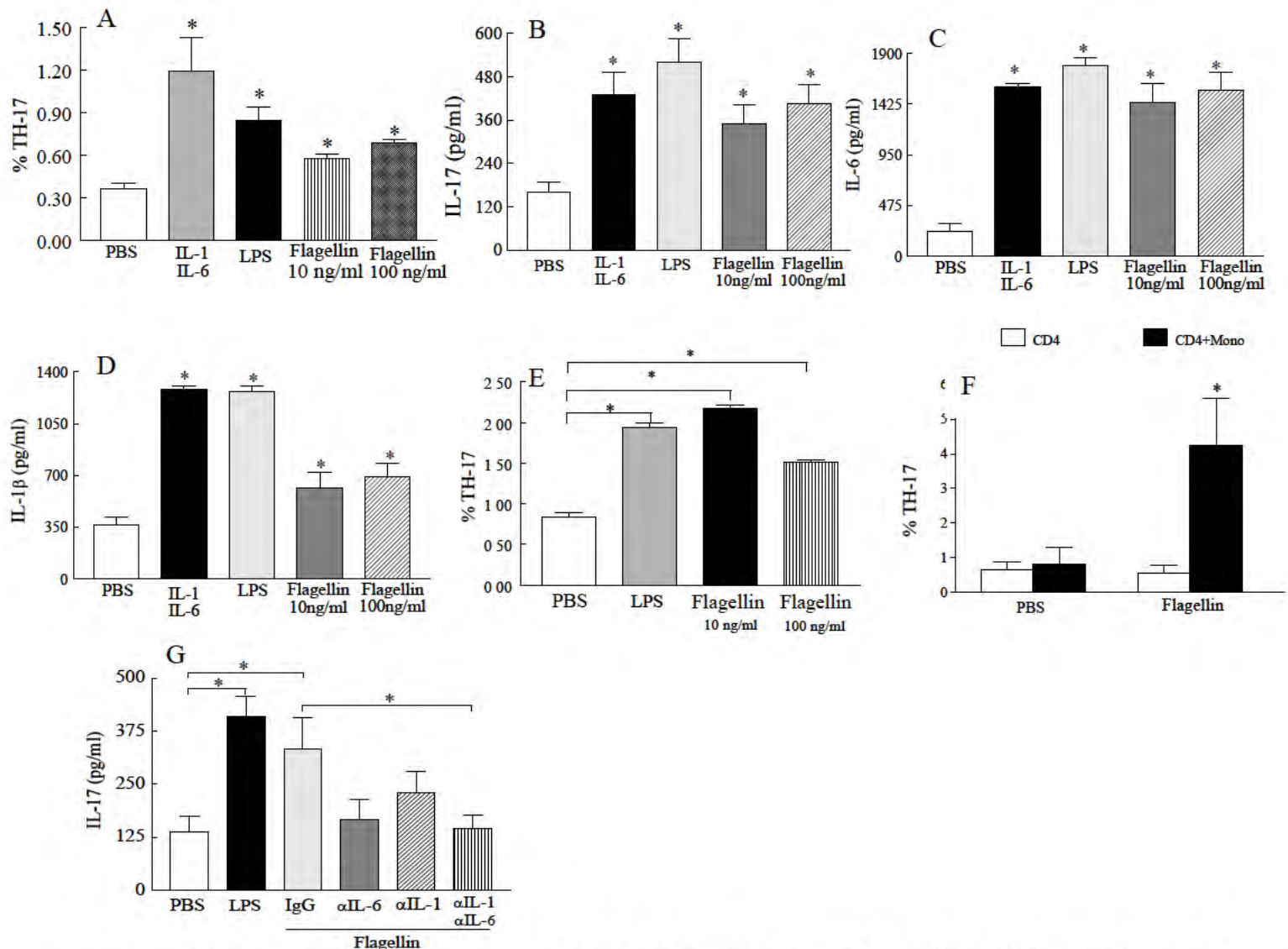

Figure 3. Like in CIA, TLR5 ligation drives TH-17 cell differentiation in normal and RA peripheral blood mononuclear cells. Normal peripheral blood mononuclear cells were either untreated or treated with LPS (100 ng/ml) or flagellin (10 and 100 ng/ml; InvivoGen) for 5 days. Cell condition media was harvested and cells were thereafter treated with 4h of PMA (50 ng/ml), ionomycin (750 ng/ml) and Brefeldin A (3 μg/ml) to quantify TH-17 cells or IL-17 and its related cytokines, n=3. **A.** Percent TH-17 cells was determined in normal peripheral blood mononuclear cells differentially treated as detailed above. Protein levels of IL-17 (**B**), IL-6 (**C**) and IL-1β (**D**) was determined in the condition media of the normal peripheral blood mononuclear cells treated as mentioned above employing ELISA, n=3. **E.** RA peripheral blood mononuclear cells were treated similar to normal cells as detailed in Fig. 4 and % TH-17 cells was determined by FACS analysis, n=3. **F.** RA CD4+ T cells and CD16+

monocytes were negatively selected and were either cultured alone or together. Subsequently cells were treated with PBS or flagellin (100 ng/ml) for 5 days and %TH-17 cells was determined by FACS analysis, n=3. **G.** Peripheral blood mononuclear cells were either untreated or treated with (10µg/ml) IgG control, anti-IL-6, anti-IL-1β or anti-IL-6 plus anti-IL-1β antibodies prior to adding flagellin (100 ng/ml) for 5 days. In these experiments LPS was used as positive control. Concentration of IL-17 in supernatants was determined by ELISA, n=3. Values are mean ± SE or data obtained from each experiment. * indicates p<0.05.

In this study we demonstrate that like TLR4, ligation of TLR5 in RA and CIA participates in TH-17 cell development by inducing expression of IL-1β and IL-6 from myeloid cells. In contrast to our observations, ligation of TLR5 in human dendritic cells did not contribute to IL-1β and IL-6 induced TH-17 development while ligation of TLR2, 3 and 4 facilitated this process (3). The discrepancy in our results may be due to several experimental differences. In our experiments, monocytes cocultured with CD4+ T cells were activated with 100 ng/ml flagellin in presence of 10% media and TH-17 cells were quantified following 5 days incubation, whereas in the previous studies dendritic cells were stimulated with 500 ng/ml flagellin for 24h prior to being cocultured with CD4+ T cells in serum free media for 7 days (3). Interestingly our previous results demonstrate that expression of TLR5 in RA macrophages and fibroblasts (cells expressed in the lining) is modulated by IL-17 (4) suggesting that there may be a cross regulation between these two cascades. Collectively these results indicate that myeloid cell ligation of TLR5 and production of TH-17 deriving factors is the initial step involved in TH-17 development and the self-perpetuating feedback regulation between the TLR5 and IL-17 pathways may contribute to perpetuation of arthritis.

TLR5 mediated IL-17 production contributes to endothelial migration.
Since in RA and CIA, TLR5 ligation strongly induces TH-17 cell polarization, we asked whether IL-17 detected in flagellin activated peripheral blood mononuclear cell condition media is capable of inducing endothelial migration. Our data show that neutralization of IL-17 in the culture media or blockade of IL-17RC on endothelial cells can greatly (40-50% respectively) reduce endothelial cell homing mediated by flagellin activated condition media (Fig. 4). Our findings suggest that TLR5 ligation on myeloid cells that are in close contact with T cells can indirectly result in IL-17 mediated vascularization.
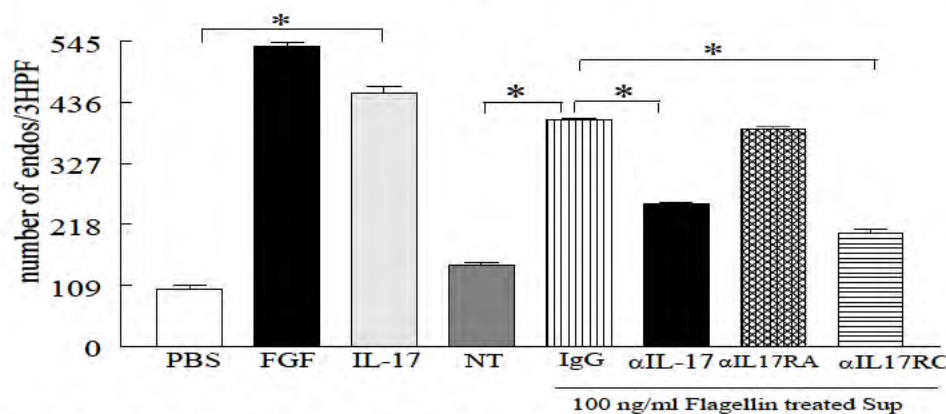
Figure 4. Blockade of IL-17 pathway reduces flagellin mediated endothelial chemotaxis. Migration of endothelial cells untreated (IgG control) or treated with antibody to IL-17RA or IL-17RC (10 µg/ml) was examined in response to flagellin (100 ng/ml) activated peripheral blood mononuclear cell supernatants. Endothelial cell chemotaxis was also determined in response to flagellin condition media immunoneutralized with anti-IL-17 or IgG control (10 µg/ml), n=3. Values are mean ± SE or data obtained from each experiment. * indicates p<0.05.

We document that ligation of TLR5 can increase CIA disease severity in part by enhancing joint vasculaization. IL-17 produced by TLR5 activation in peripheral blood mononuclear cells was capable

of attracting endothelial cells via IL-17RC but not IL-7RA ligation confirming the previously reported results (5). Interestingly both TLR5 and IL-17 mediated endothelial chemotaxis and tube formation are regulated via PI3K/AKT1 pathway (5) suggesting an overlapping mechanism of action. However recent data demonstrate that similar to TLR5, angiogenesis mediated by IL-17 is induced directly via IL-17RC on endothelial cells (5) and/or indirectly through induction of CXCL5 from RA synovial tissue fibroblasts and myeloid cells (6). Therefore induction of TH-17 cells/IL-17 may amplify TLR5 mediated CIA vascularization by increasing CXCL5 production that mediates angiogenesis through activation of NF-κB which is an IL-17 and TLR5 non overlapping cascade (6).

In conclusion we demonstrate for the first time that ligation of TLR5 on endothelial cells can directly contribute to RA neovascularization and further TLR5 agonist can enhance RA and CIA disease severity by elevating TH-17 polarization and IL-17 mediated angiogenesis. We also found the TLR5 is the link between IL-17 produced from TH-17 cells and TNF secreted from myeloid cells.

TASK 3 : To examine the indirect role of IL-17 in mediating angiogenesis and arthritis. We will: **(time frame, months 24-36).**

3a. Examine the mechanism by which IL-17-induced angiogenesis is associated with VEGF. (time frame, months 24-26).

A summary of the 2013 progress report: Angiogenesis is an early and a critical event that fosters chronic inflammation and bone erosion in RA by facilitating unbalanced leukocyte migration and pannus formation. Hence inhibition of angiogenesis may lead to identifying novel therapeutic approaches for RA. Macrophages are hypoxia sensors that initiate and maintain angiogenesis in RA synovium. We found that CCR7 was the most highly upregulated gene in macrophages obtained from RA synovial fluid compared to the normal myeloid cells by microarray analysis. In validation of the microarray data, macrophages in the RA synovial tissue lining and endothelial cells in the sublining express elevated levels of CCR7 and its ligand CCL21. We uncovered that synovial CCL21 but not CCL19 is a novel and potent chemoattractant for CCR7+ endothelial cells, which plays a pivotal role in RA tube and blood vessel formation. In RA synovial tissue explants, CCL21 driven angiogenesis can be also induced indirectly through VEGF production. Interestingly we uncovered that CCL21 is the missing link between IL-17 and VEGF mediated vascularization as blockade of CCL21 function markedly suppresses IL-17 induced VEGF expression in RA ST fibroblasts.

3b and 3c. Investigate whether IL-17 angiogenesis *in vivo* is due to down stream proangiogenic factors. (time frame, months 26-36).

A summary of the 2012 progress report: Results from our previous studies demonstrate that IL-17 is a potent proangiogenic factor in RA that can facilitate neovascularization directly through IL-17RC ligation (5). Findings from our (6) and other laboratories indicate that they may be factors downstream IL-17 that also contribute to RA angiogenesis.

To demonstrate whether there are indirect pathways associated with IL-17 induced angiogenesis, we examined potent proangiogenic factors induced by IL-17 in RA synovial tissue as well as in IL-17 experimental arthritis model. We found that expression of CXCL1 and CXCL5 was highly elevated by IL-17 in RA synovial tissue explants and animal models of RA. To demonstrate the pathologic role of CXCL1 and CXCL5 in IL-17 mediated arthritis, neutralizing antibodies to each chemokine were employed. We found that arthritis severity and vascularization were significantly reduced in the anti-CXCL5 treatment group. In contrast, anti-CXCL1 treatment had no effect on IL-17 mediated disease activity or neovascularization, while being capable of inhibiting CXCL1 mediated endothelial chemotaxis *in vitro*. The combination of anti-CXCL1 and anti-CXCL5 was not more effective than

anti-CXCL5 treatment alone. We next demonstrated that ligation to CXCR2 facilitates CXCL1 and CXCL5 induced endothelial migration although downstream signaling pathways are differentially regulated by these chemokines. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF-κB signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF-κB mediated angiogenesis. In conclusion, these observations suggest that IL-17 mediated joint vascularization may be in part due to CXCL5 induction. These findings are now published in Angiogenesis 2011; 14: 443-455 (6).

KEY RESEARCH ACCOMPLISHMENTS

Within this short time frame we have shown that:

- RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 and IL-17 does not synergize with VEGF in RA synovial fluid induced HMVEC migration.

- Neutralization of IL-17RC but not IL-17RA was involved with suppressing RA synovial fluid-mediated HMVEC migration.

- Activation of PI3K is responsible for IL-17-mediated HMVEC tube formation and migration.
- Expression of CXCL1 and CXCL5 is highly elevated in RA synovial tissues treated with IL-17 and in IL-17 induced arthritis model while VEGF was not markedly increased in any of the mentioned models.
- Inhibition of CXCL5 but not CXCL1 relieves IL-17-induced arthritis.
- Anti-CXCL5 reduces levels of joint TNF-α and vascularization in IL-17-induced arthritis model.
- CXCL5 but not CXCL1 induces endothelial migration and angiogenesis through an IL-17 non overlapping mechanism.
- IL-17 mediated joint vascularization is in part due to CXCL5 induction.
- CCL21 ligation to CCR7 connects IL-17 and VEGF induced neovascularization.
- Disruption of CCL21 binding to CCR7 markedly suppresses IL-17 mediated VEGF transcription.
- IL-17 modulates expression of CCL21 and CCR7 in human endothelial cells.
- CCL21 synergizes with VEGF in promoting endothelial migration.
- Local expression of CCL21 promotes joint inflammation and neovascularization.
- IL-17 regulates expression of CCL28 in RA monocytes and RA peripheral blood in vitro differentiated macrophages
- CCL28 is expressed in in RA synovial fluid and strongly promotes to RA angiogenesis through CCR10 ligation and ERK activation.
- In RA activation of TLR5 links TND and IL-17 cascades
- In RA and preclinical models of RA, TLR5 induces angiogenesis in part by TH-17 cell / IL-17 induction

CONCLUSION

In RA patients percent TH-17 cells correlate with disease activity score suggesting that IL-17 can mediate inflammation at early disease stage and may also be involved in disease progression (7). Therefore to evaluate the role of IL-17 in RA pathogenesis, we examined which IL-17 receptors and signaling pathways are associated with rheumatoid arthritis synovial fluid mediated endothelial migration and tube formation. We show that although HMVECs express both IL-17RA and RC, RA

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synovial fluid-mediated HMVEC chemotaxis is mediated by binding primarily to IL-17RC. We also report that while IL-17 activates ERK, JNK and PI3K pathways in HMVECs, only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation. We show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone.

To demonstrate whether there are indirect pathways associated with IL-17 induced angiogenesis, we examined potent proangiogenic factors induced by IL-17 in RA synovial tissue as well as in IL-17 experimental arthritis model. We found that expression of CXCL1 and CXCL5 but not VEGF was highly elevated by IL-17 in RA synovial tissue explants and animal models of RA. To demonstrate the pathologic role of CXCL1 and CXCL5 in IL-17 mediated arthritis, neutralizing antibodies to each chemokine were employed. We found that arthritis severity and vascularization were significantly reduced in the anti-CXCL5 treatment group. In contrast, anti-CXCL1 treatment had no effect on IL-17 mediated disease activity or neovascularization, while being capable of inhibiting CXCL1 mediated endothelial chemotaxis *in vitro*. The combination of anti-CXCL1 and anti-CXCL5 was not more effective than anti-CXCL5 treatment alone. We next demonstrated that ligation to CXCR2 facilitates CXCL1 and CXCL5 induced endothelial migration although downstream signaling pathways are differentially regulated by these chemokines. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF-κB signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF-κB mediated angiogenesis. In conclusion, these observations suggest that IL-17 mediated joint vascularization may be in part due to CXCL5 induction.

Production of IL-17 from joint TH-17 cells can strongly contribute to RA angiogenesis (5) through a mechanism that is in part due to induction of VEGF from RA ST fibroblasts (8, 9). We document that CCL21 is expressed from endothelial cells activated by IL-17 (10) and neutralization of CCL21 markedly reduces IL-17 mediated VEGF transcription from the RA ST. Like IL-17, CCL21 is also capable of enhancing production of VEGF from RA ST fibroblasts and can further synergize with VEGF in facilitating endothelial chemotaxis. Hence CCL21 may be the unidentified connecting factor between the IL-17 and VEGF cascades. Therapeutic targeting of VEGF and VEGFR has led to disappointing results regarding drug toxicity and lack of efficacy in patients with advanced tumor growth (11, 12) therefore RA patients were not treated with anti-VEGF or anti-VEGFR therapies. However, since we demonstrate that CCL21 induced by IL-17 can modulate VEGF expression in RA ST, targeting CCL21 may disconnect the link between IL-17 and VEGF cascade and therefore more efficiently suppress RA neovascularization.

We show for the first time that expression of CCL28 and CCR10 is markedly higher in RA and OA ST lining macrophages and sublining endothelial cells compared to NL ST. We found that expression of CCL28 in RA myeloid cells is modulated by IL-17. We uncovered that CCL28 strongly attracts endothelial cells at the physiological concentration available in RA SF. Last, we document that knockdown of endothelial CCR10 significantly reduces CCL28 mediated endothelial migration and capillary formation through an ERK dependent mechanism.

Progress report 2015: We discovered that local expression of IL-17 promotes joint inflammation through elevated vascularization; however the upstream cascade that modulates IL-17 driven pathology is undefined in rheumatoid arthritis (RA). Therefore the impact of TLR5 ligation was examined on TH-17 cell polarization and joint vascularization *in vivo* in chronic and acute arthritis models and *in vitro* in RA peripheral blood mononuclear cells (PBMC)s and endothelial cells. We uncovered that when collagen induced arthritis (CIA) mice were therapeutically treated with the TLR5 ligand, flagellin, joint inflammation was greatly increased while the control ankle swelling remained at a plateau phase. We

show that joint endothelial staining, hemoglobin concentration, ankle IL-17 and spleen TH-17 cells were higher in the flagellin group compared to the controls. Examination of the underlying mechanism using RA PBMCs showed that ligation of TLR5 to myeloid cells and production of IL-6 and IL-1 is essential for TH-17 polarization and that CD4+ T cells alone were incapable of this function. We further demonstrate that neutralization of IL-17 dysregulates endothelial cell migration activated by flagellin conditioned medium, indicating that TLR5 mediated angiogenesis is in part due to TH-17 differentiation. We conclude that in RA, TH-17 cell maturation and function is interconnected to TLR5 cascade, hence blockade of TLR5 may impair IL-17 driven arthritis.

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1. UIC http://news.uic.edu/molecules-involved-in-rheumatoid-arthritis-angiogenesis-identified
2. Arthritis Research UK http://www.arthritisresearchuk.org/news/general-news/2014/may/new-protein-discovery-sheds-light-on-rheumatoid-arthritis-progression.aspx
3. Science News Line Medicine http://www.sciencenewsline.com/articles/2014051615180018.html
4. National Pain report http://americannewsreport.com/nationalpainreport/two-proteins-linked-to-rheumatoid-arthritis-8823861.html
5. Science Daily http://www.sciencedaily.com/releases/2014/05/140516111003.htm
6. Arthritis Digest UK http://arthritisdigest.co.uk/potential-avenue-of-treatment-for-rheumatoid-arthritis-thanks-to-discovery-of-a-new-protein/
7. US daily news http://www.dailynewsen.com/science/molecules-involved-in-rheumatoid
8. Science Daily   Article: http://www.sciencedaily.com/releases/2014/05/140516111003.htm
9. Bioscience Technology http://www.biosciencetechnology.com/news/2014/05/two-proteins-play-key-role-rheumatoid-arthritis
10. Protein Products online http://www.ptproductsonline.com/2014/05/protein-molecules-part-rheumatoid-arthritis-angiogenesis-identified/
11. Orthopedics this week http://ryortho.com/breaking/ra-molecules-involved-in-angiogenesis-identified/

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myeloid cell infiltration and differentiation to mature osteoclasts in RA patients. J Immunol., 15;193:3902-3913, 2014.

22. Nature Review Rheumatology highlights our paper and comments on us discovering **A TLR5–TNF positive feedback loop in rheumatoid arthritis.** Bernard NJ. *Nature Rev Rheumatology* 10, 637 (2014) doi:10.1038/nrrheum.2014.166 Published online 23 September 2014.

Additionally our paper was highlighted in *National Institute of Arthritis and Musculoskeletal and Skin Disease (NIAMS)* website (please see the second link below)

Findings highlighted in:
1. NIH/NIAMS https://www.facebook.com/NIH.NIAMS?sk=app_139229522811253
2. Nature Review Rheumatology
http://www.nature.com/nrrheum/journal/vaop/ncurrent/full/nrrheum.2014.166.html
3. UIC http://news.uic.edu/blocking-one-receptor-on-cells-could-halt-rheumatoid-arthritis
4. Medical News Today http://www.medicalnewstoday.com/articles/282376.php
5. Science Daily http://www.sciencedaily.com/releases/2014/09/140910102826.htm
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9. University of Illinois board of Trustees http://www.veooz.com/news/NHU38A6.html
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ABSTRACTS AND ORAL PRESENTATIONS:

1. Pickens SR, Volin MV, Mandelin II AM, **Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis. Arthritis Rheum 714: S298, 2010. **Oral presentation**

2. Pickens SR, Volin MV, Pope RM, Mandelin II AM, **Shahrara S**. Characterization of CCR7 and its ligands CCL19 and CCL21 in rheumatoid arthritis. Arthritis Rheum 1506: S627, 2010.

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6. Chamberlain ND, Pickens SR, Pope RM, Volin MV, **Shahrara S**. IL-7 Contributes to Monocyte Migration in Rheumatoid Arthritis. Arthritis Rheum 729: S284, 2011. **Oral presentation**.

7. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM II, **Shahrara S**. CCL21 a Novel Proangiogenic Factor in Rheumatoid Arthritis. Arthritis Rheum 730: S284, 2011. **Oral presentation**.

8. Chamberlain ND, Volin MV, Pope RM, Mandelin AM II, **Shahrara S**. Characterization of TLR7 and TLR8 in Rheumatoid Arthritis. Arthritis Rheum 364: S136, 2011.

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Abstract for Military Health System Research Symposium (MHSRS) 2014:

17. Kim SJ, Chen Z, Chamberlain ND, Volin MV, Essani AB and *Shahrara S. Identifying a novel cascade that modulates IL-17 driven arthritis. Military Health System Research Symposium 2014 in Fort Lauderdale, FL.

INVITED LECTURES:

2010	University of Illinois in Chicago, Rheumatology rounds
2010	American College of Rheumatology Research and Education Foundation *Within Our Reach* meeting in Dallas
2010	Northwestern University, Feinberg School of Medicine, Pathology and inflammation group
2010	American College of Rheumatology 2010 annual meeting held in Atlanta
2011	Department of Microbiology and Immunology lectures in University of Illinois at Chicago
2011	The Institute for Personalized Respiratory Medicine lectures in University of Illinois at Chicago
2011	Department of Rheumatology Grand Rounds in University of Illinois at Chicago
2011	American College of Rheumatology 2011 annual meeting held in Chicago
2012	Department of Microbiology and Immunology lectures in University of Illinois at Chicago
2012	Department of Rheumatology Grand Rounds in University of Illinois at Chicago
2012-2013	Moderator for ACR abstract session "Cytokines, Mediators, Cell-cell adhesion, Cell trafficking and Angiogenesis"
2012	American College of Rheumatology 2012 annual meeting plenary session held in Washington D.C.
2013	EULAR 2013 held in Spain, Chemokines in monocyte endothelial interactions

2013	Invited speaker in Pulmonary Hypertension Seminar Series in University of Illinois at Chicago
2013	Lecturing Rheumatology fellows on the role of cytokines in rheumatoid arthritis
2013	Invited speaker to Rheumatology Grand Rounds at University of Illinois at Chicago
2013	Invited speaker in UICenter Seminar Series in University of Illinois at Chicago
2014	Invited speaker to Grand Rounds at New York University
2014	Invited speaker to Rheumatology Grand Rounds at University of Illinois at Chicago
2014	American College of Rheumatology 2014 annual meeting held in Boston
2015	University of Illinois Grand Rounds
2015	Cleveland Clinic Grand Rounds

INVENTIONS, PATENTS AND LICENSES

None

REPORTABLE OUTCOMES

Through this funding we have published 22 papers in high impact factor journals. We have also presented 17 abstracts or oral presentations in American College of Rheumatology and Military Health System Research Symposium. Further we have identified a novel target for RA treatment which is upstream of IL-17 cascade. We recently received the University of Illinois Chancellor's Innovation Fund Proof-of-Concept Awards to make a fully human antibody as well as a human:mouse chimera antibody to test the efficacy of the identified novel target in human cells and animal models of RA. We also constructed two adenoviruses that express mouse CCL21 and CCL28 which are IL-17 downstream pro-angiogenic factors that play a critical role in RA pathogenesis.

OTHER ACHIEVEMENTS

The funding provided to us by DOD enabled me to secure a tenure track position as an Associate Professor of Medicine in University of Illinois at Chicago (UIC). My position started at March 1st of 2011 and the funding provided by DOD was the basis of obtaining this opportunity. I am humbled and grateful for receiving this Investigator Initiated Award from the Department of Defense. Funding from DOD has enabled us to publish 24 papers in high impact factor Journals in short time frame (including an invited Nature Review Rheumatology paper). We are currently preparing 3 more manuscripts which will be submitted before the end of the year. Additionally through the funding available to us from DOD we have presented 7 oral and 7 poster presentations. In endorsement of our productive cutting edge research our most recent findings pertaining to the role of IL-17 in RA angiogenesis was accepted for oral presentation at ACR discoveries of 2012 plenary session. In short as a result of DOD funding, I

have established my own independent laboratory and have made great discoveries in finding potential treatment for patients that suffer from RA. The DOD funding also allowed me to receive a VA Merit award and become tenure associate professor of medicine in 2015. I am very grateful for obtaining this funding.

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APPENDICES

1. Our paper published in J Immunol, 2010, 184:3233-3241, supports our finding presented in this 2014 progress report. Submitted before.

2. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM 2nd, Agrawal H, Matsui M, Yoshimoto T, *Shahrara S. Local expression of IL-27 ameliorate collagen induced arthritis. Arthritis Rheum 2011, 63:2289-98. Supports the role of IL-7 and TH-17 in RA angiogenesis. Submitted before.

3. Pickens SR, Chamberlain ND, Volin MV, Gonzalez M, Pope RM, Mandelin AM II, Kolls JK, *Shahrara S. Anti-CXCL5 therapy ameliorates IL-17-induced arthritis by decreasing joint vascularization. Angiogenesis. 2011,14:443-455. Supports our progress report. Submitted before.

4. Chamberlain ND, Vila OM, Volin MV, Volkov S, Pope RM, Swedler W, Mandelin AM 2nd, *Shahrara S. TLR5, a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis that Correlates with Disease Activity Score and Joint TNF-α Levels. J Immunol. 2012. Submitted before.

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6. Chen Z , Kim S, Essani AB, Volin MV, Vila OM, Swedler W, Arami A, Volkov S, Sweiss S and Shahrara S*. Characterizing the expression and function of CCL28 and its corresponding receptor CCR10 in RA pathogenesis. Annals Rheum Dis, 2014 In press. Supports the role of CCL28 in IL-17 mediated angiogenesis. Submitted before.

7. Kim SJ, Chen Z, Chamberlain ND, Volin MV, Swedler W, Volkov S, Sweiss N, ***Shahrara S**. Angiogenesis in rheumatoid arthritis is directly fostered by TLR5 ligation and indirectly through IL-17 induction. Arthritis Rheum 65: 2024-2036, 2013. Submitted in 2015 progress report.

8. Nature Review Rheumatology highlights our paper and comments on us discovering **A TLR5–TNF positive feedback loop in rheumatoid arthritis.** Bernard NJ. *Nature Rev Rheumatology* 10, 637 (2014) doi:10.1038/nrrheum.2014.166 Published online 23 September 2014. Submitted in 2015 progress report.

9. Kim SJ, Chen Z, Chamberlain ND, Essani AE, Volin MV, Volkov S, Amin AM, Gravallese EM, Arami S, Swedler W, Lane NE, Mehta A, Sweiss N, ***Shahrara S**. Ligation of TLR5 promotes myeloid cell infiltration and differentiation to mature osteoclasts in RA patients. J Immunol., 15;193:3902-3913, 2014. Submitted in 2015 progress report.

Angiogenesis in Rheumatoid Arthritis Is Fostered Directly by Toll-like Receptor 5 Ligation and Indirectly Through Interleukin-17 Induction

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Objective. To examine the impact of Toll-like receptor 5 (TLR-5) on endothelial cell function in rheumatoid arthritis (RA) and vascularization in collagen-induced arthritis (CIA).

Methods. Endothelial cell migration and tube formation assays were used to demonstrate the direct role of TLR-5 ligation in angiogenesis. Mice with CIA were treated with the TLR-5 agonist flagellin to document the effect of TLR-5 ligation in RA pathology. Vascularization in CIA was determined by immunohistochemical analysis and determination of cytokine levels in ankle joints. Spleen Th17 cells and joint interleukin-17 (IL-17) were quantified by fluorescence-activated cell sorting analysis and enzyme-linked immunosorbent assay. The development of Th17 cells induced by TLR-5 ligation was validated in RA peripheral blood mononuclear cells.

Results. Ligation of TLR-5 to endogenous ligands expressed in RA synovial fluid contributed to endothelial cell infiltration and tube formation. Furthermore, treatment with flagellin after the onset of CIA exacerbated joint inflammation; in contrast, inflammation in control mice remained at a plateau phase. We showed that TLR-5–enhanced disease severity was attributable to Th17 cell differentiation and joint vascularization in CIA. Examination of the underlying mechanism using RA peripheral blood mononuclear cells documented that ligation of TLR-5 in myeloid cells and production of Th17–promoting cytokines were necessary for Th17 cell polarization. Additionally, we demonstrated that blockade of the IL-17 cascade markedly reduced endothelial cell migration activated by flagellin-conditioned medium, suggesting that TLR-5 ligation can mediate RA angiogenesis either directly by attracting endothelial cells or indirectly by fostering Th17 cell development.

Conclusion. Our data demonstrate a novel role for TLR-5 in RA angiogenesis; thus, TLR-5 may be a promising new target for RA treatment.

Rheumatoid arthritis (RA) is a chronic autoimmune disease in which Toll-like receptor (TLR)–activated innate immunity has an important role (1,2). Previous studies documented the expression of TLR-2 and TLR-4 in RA peripheral blood, tissue, and synovial fluid (SF) (3–5). Furthermore, the significance of these receptors in the pathogenesis of experimental models of both acute and chronic arthritis was demonstrated (6). This specific interest in TLR-2 and TLR-4 was initiated by the identification of endogenous ligands for these receptors in RA, which include fibrinogen, Hsp60, Hsp70, and Hsp96 as well as fibronectin extra domain A (7). Ligation of TLR-2 and TLR-4 has been associated with the production of proinflammatory cytokines, chemokines, destructive matrix metalloproteinases, and proangiogenic factors in RA (1), suggesting that TLR-2/4 activation can contribute to RA pathogenesis by inducing leukocyte migration, bone erosion, and angiogenesis (1,8,9).

We recently demonstrated that the expression of TLR-5 in synovial tissue lining and sublining macro-

phages and endothelial cells from patients with RA and patients with osteoarthritis is elevated compared with that in normal individuals (10). We documented that endogenous TLR-5 ligand(s) are present in RA SF, and that ligation of TLR-5 in RA myeloid cells can promote the production of several proinflammatory factors (10). The significance of expression of TLR-5 by myeloid cells in the pathogenesis of RA was validated by demonstrating a strong correlation between the Disease Activity Score in 28 joints (11) and concentrations of TLR-5 and tumor necrosis factor α (TNFα) in RA monocytes (10). A previous study by our group focused on the role of TLR-5 in RA myeloid cells; however, the impact of TLR-5 on endothelial cell function in RA or collagen-induced arthritis (CIA) is completely undefined.

Angiogenesis is an early and critical event in RA that promotes leukocyte infiltration and pannus formation, thereby perpetuating chronic inflammation (12–14). Interestingly, neovascularization mediated by TLR-2 and TLR-4 ligation has been shown to be attributable to the secretion of several proangiogenic factors, such as interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and TNFα, from RA synovial tissue fibroblasts and/or myeloid cells (9,15). Other investigators have shown that TLR-2–induced angiogenesis and cell invasion are in part due to secretion of angiopoietin 2 from RA synovial explants and binding to Tie2 expressed on synovial sublining endothelial cells (16). Other findings demonstrate that lipopolysaccharide (LPS)–induced activation of the NF-κB pathway is responsible for hypoxia-inducible factor 1α–activated VEGF production in macrophages (17). These results suggest that angiogenesis triggered by TLR-2 and TLR-4 ligation is indirectly mediated via production of potent proangiogenic factors.

Here, we demonstrate for the first time that TLR-5 ligation induces endothelial cell chemotaxis and tube formation through activation of the phosphatidylinositol 3-kinase (PI3K)/Akt-1 pathway. We also show that, as in RA, expression of TLR-5 in CIA is increased in lining and sublining macrophages and endothelial cells. We document that treatment with TLR-5 agonist after the onset of CIA enhances disease severity by promoting Th17 cell differentiation as well as joint neovascularization. Last, we show that differentiation of Th17 cells and production of IL-17 (also known as IL-17A) in the joint are interconnected with TLR-5–mediated angiogenesis, because blockade of the IL-17 cascade can significantly suppress endothelial cell chemotaxis in response to TLR-5 agonist–conditioned medium. Our novel results suggest that TLR-5 ligation

mediates RA angiogenesis either directly or indirectly through Th17 cell development. These results suggest that disruption of TLR-5 function may suppress the pathogenesis of RA.

MATERIALS AND METHODS

Endothelial cell chemotaxis. For the assessment of chemotaxis, human microvascular endothelial cells (HMVECs) and/or human umbilical vein endothelial cells (HUVECs) (Lonza) were incubated in endothelial basal medium (Lonza) with 0% fetal bovine serum for 2 hours before being used. HMVECs/HUVECs from different experiments (Figures 1A–C and F and Figure 6C) were then placed in the bottom wells of a 48-well Boyden chemotaxis chamber (Neuro Probe) (18,19). Following incubation for 2 hours at 37°C, the membranes were removed, fixed, and stained.

To investigate whether flagellin ligation to TLR-5 could contribute to endothelial cell migration, we examined endothelial cell extravasation in response to flagellin (Ultrapure; endotoxin level <50 EU/mg) (InvivoGen) at concentrations of 0.001–100 ng/ml. Phosphate buffered saline (PBS) was used as a negative control, and VEGF (10 ng/ml; R&D Systems) was used as a positive control. To determine whether endogenous TLR-5 ligands are present in RA SF and whether their ligation to TLR-5 could contribute to RA SF–mediated endothelial cell trafficking, HMVEC/HUVEC chemotaxis induced by RA SFs (1:20 dilution) was examined following a 1-hour incubation of cells with control IgG or anti–TLR-5 antibody (10 μg/ml; InvivoGen).

The signaling pathway(s) that contribute to endothelial cell chemotaxis in response to flagellin (10 ng/ml) were defined by incubating HMVECs/HUVECs with chemical inhibitors (concentration 1 μM or 5 μM) to PI3K (LY294002), ERK (PD98059), p38 (SB203580), NF-κB (BAY 11-7082) (all from EMD Millipore), or DMSO for 2 hours in a Boyden chamber. To determine whether flagellin-mediated IL-17 production by peripheral blood mononuclear cells (PBMCs) is responsible for endothelial cell chemotaxis, we examined the migration of endothelial cells that were untreated (IgG control) or treated with neutralizing antibodies to IL-17RA or IL-17RC (10 μg/ml; R&D Systems) in response to IL-17 (2.5 ng/ml, the highest concentration of IL-17 in flagellin-activated conditioned medium) or in PBMC supernatants activated with flagellin (100 ng/ml). Endothelial cell chemotaxis in response to flagellin-activated conditioned medium or IL-17 immunoneutralized with anti–IL-17 or IgG control (10 μg/ml; R&D Systems) was also determined.

Tube formation. For the Matrigel tube formation assay, BD Matrigel matrix (BD BioCoat Tube Formation kit; 50 μl) was polymerized for 30 minutes at 37°C in a 96-well plate. In order to determine whether TLR-5 ligation is pivotal for RA SF–induced endothelial cell tube formation, HMVECs/HUVECs were incubated with antibodies to TLR-5 or IgG (10 μg/ml) for 45 minutes at 37°C. The pretreated cells plus RA SF (1:20) or flagellin (10 ng/ml) were added to the polymerized Matrigel, and the plate was incubated for 16 hours at 37°C. Each condition was performed in triplicate; basic fibroblast growth factor (20 ng/ml; R&D Systems) was used as

a positive control, and PBS was used as a negative control. Following incubation, the number of branch points/tubes was quantified as previously described (18–21).

Flagellin signaling in endothelial cells. HMVECs/HUVECs were left untreated or were treated with flagellin (10 ng/ml) for 15–65 minutes. Cell lysates were analyzed by Western blotting, as previously described (18,22). Blots were probed with phospho–Akt-1, phospho-ERK, phospho-p38 (1:1,000 dilution; Cell Signaling Technology), and degradation of IκB (Santa Cruz Biotechnology; 1:3,000 dilution) overnight or were probed with Akt, ERK, p38, or actin (1:3,000 dilution; Cell Signaling Technology or Sigma).

Study protocol for CIA induction and flagellin treatment. Male DBA/1 mice (7–8 weeks old) were immunized on days 0 and 21 with bovine type II collagen (2 mg/ml; Chondrex) emulsified in equal volumes of Freund's complete adjuvant (2 mg/ml; Chondrex); 100 μl of emulsion was administered subcutaneously in the tail. On day 21, the mice were injected intradermally with 100 μl of type II collagen (2 mg/ml) emulsified in equal volumes of Freud's incomplete adjuvant (23). Flagellin (20 μg; InvivoGen) (n = 10) or PBS (n = 10) was injected intraperitoneally on day 33 after the induction of CIA. The mice were killed on day 57, and the ankles were harvested for protein and messenger RNA extraction as well as histologic studies; sera were saved for laboratory testing. Flagellin treatment post–CIA onset was performed twice, and the clinical and histologic results were very consistent. In a different experiment, CIA was induced as described above on days 0 and 21, and the ankles were harvested on day 45 post–CIA induction. Subsequently, TLR-5 immunostaining was compared in the ankles of mice with CIA and PBS-injected mice.

Clinical assessments. Ankle circumference was determined by caliper using the following formula: circumference = $2\pi \times (\sqrt{[a^2 + b^2/2]})$, where a and b represent the laterolateral and anteroposterior diameters, respectively. For studies of mice with CIA, ankle circumference evaluations were performed on days 4, 19, 21, 26, 28, 33, 35, 37, 40, 42, 44, 47, 49, 51, and 54 (23).

Antibodies and immunohistochemical analysis. Mouse ankles were decalcified, formalin fixed, paraffin embedded, and sectioned. Inflammation, synovial lining thickness, and bone erosion (scale of 0–5) were determined by 2 observers in a blinded manner, using hematoxylin and eosin–stained sections. For TLR-5 and von Willebrand factor (vWF) immunostaining, slides were deparaffinized in xylene, followed by rehydration by transfer through graded alcohols. Antigens were unmasked by incubating slides in proteinase K digestion buffer (Dako). Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2. Nonspecific binding of avidin and biotin was blocked using an avidin–biotin blocking kit (Dako). Nonspecific binding of antibodies to the tissue was blocked by pretreatment with Protein Block (Dako). The tissue samples were incubated with antibodies to TLR-5 (1:50; Santa Cruz Biotechnology), vWF (1:1,000; Dako), or control IgG (Beckman Coulter). Staining was scored on a 0–5 scale by 2 observers (MV and SK) under blinded conditions. The data were pooled, and the mean $\pm$ SEM was calculated for each data group.

Quantification of hemoglobin in mouse ankles. Using methemoglobin, serial dilutions were prepared to generate a standard curve from 70 gm/dl to 1.1 gm/dl (18,19). Fifty

microliters of homogenized mouse ankle or standard was added to a 96-well plate in duplicate, and 50 μl of tetramethylbenzidine was added to each sample. The plate was allowed to develop at room temperature, and the reaction was terminated with 150 μl of 2N H_2SO_4 for 3–5 minutes. Absorbance at 450 nm was read with an enzyme-linked immunosorbent assay (ELISA) plate reader.

Quantification of proinflammatory factors. Mouse ankle tissue and/or serum IL-17, IL-1β, IL-6, CCL20, and VEGF expression was quantified by ELISA (R&D Systems) according to the manufacturer's instructions. Additionally, levels of human IL-17, IL-6, and IL-1β in the cell-conditioned medium obtained from normal and/or RA peripheral blood in various experiments were determined by ELISA (R&D Systems) according to the manufacturer's instructions.

Th17 cell fluorescence-activated cell sorting (FACS) analysis in splenocytes from mice with CIA and human PBMCs. To quantify the steady-state percentage of Th17 cells in mice with CIA, splenocytes were stimulated with phorbol myristate acetate (PMA; 5 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (3 μg/ml) for 4 hours. Splenocytes were then washed, blocked using anti-mouse CD16/CD32, and stained with allophycocyanin (APC)–conjugated anti-CD4 antibodies (eBioscience). Following CD4 staining, cells were fixed and permeabilized using an IC-Flow Kit (Imgenex) according to the manufacturer's instructions. Subsequently, splenocytes were stained with phycoerythrin (PE)–labeled anti–IL-17 antibodies (eBioscience), and the total number of Th17 cells per spleen was calculated.

Next, the effect of flagellin treatment on human Th17 cell polarization was examined. For this purpose, normal or RA PBMCs were isolated by Histopaque gradient centrifugation (Sigma-Aldrich) (24,25). Thereafter, normal and RA PBMCs (4 $\times$ 10^6) were cultured in anti-CD3 antibody (500 ng/ml), and cells were either left untreated or were treated with a mixture of 10 ng/ml of IL-6, IL-1β, and IL-23 and 5 ng/ml of transforming growth factor β (TGFβ) (non-TLR–positive control), LPS (TLR-specific positive control; 100 ng/ml) (InvivoGen), or flagellin (10 ng/ml and 100 ng/ml). After 5 days, culture medium was harvested, and the cells were subsequently treated for 4 hours with PMA (50 ng/ml), ionomycin (750 ng/ml), and brefeldin A (3 μg/ml) for FACS analysis of Th17 cells and/or quantification of IL-17 in cell-conditioned medium without brefeldin A by ELISA. Human Th17 cells were determined by cell-surface staining of APC-conjugated anti-CD4 antibodies (eBioscience) and intracellular staining of PE-labeled anti–IL-17 antibodies (eBioscience), as described above.

To determine whether cell-to-cell contact of monocytes with CD4+ T cells is essential for flagellin-mediated Th17 cell differentiation in RA, CD4+ T cells and CD14+ monocytes (StemCell Technologies) were negatively selected from RA PBMCs, according to the manufacturer's instructions (24). Subsequently, either RA CD4+ T cells (1 $\times$ 10^6) or CD14+ monocytes (1 $\times$ 10^6) were cultured alone or together (1:1 ratio; 1 $\times$ 10^6), and cells were maintained in a mixture of anti-CD3 antibody (2 μg/ml), anti-CD28 antibody (2 μg/ml), and recombinant IL-2 (200 units/ml) and treated with PBS or flagellin (100 ng/ml) for 5 days. The percentage of Th17 cells or the concentration of IL-17 in supernatants was determined by FACS analysis or ELISA.

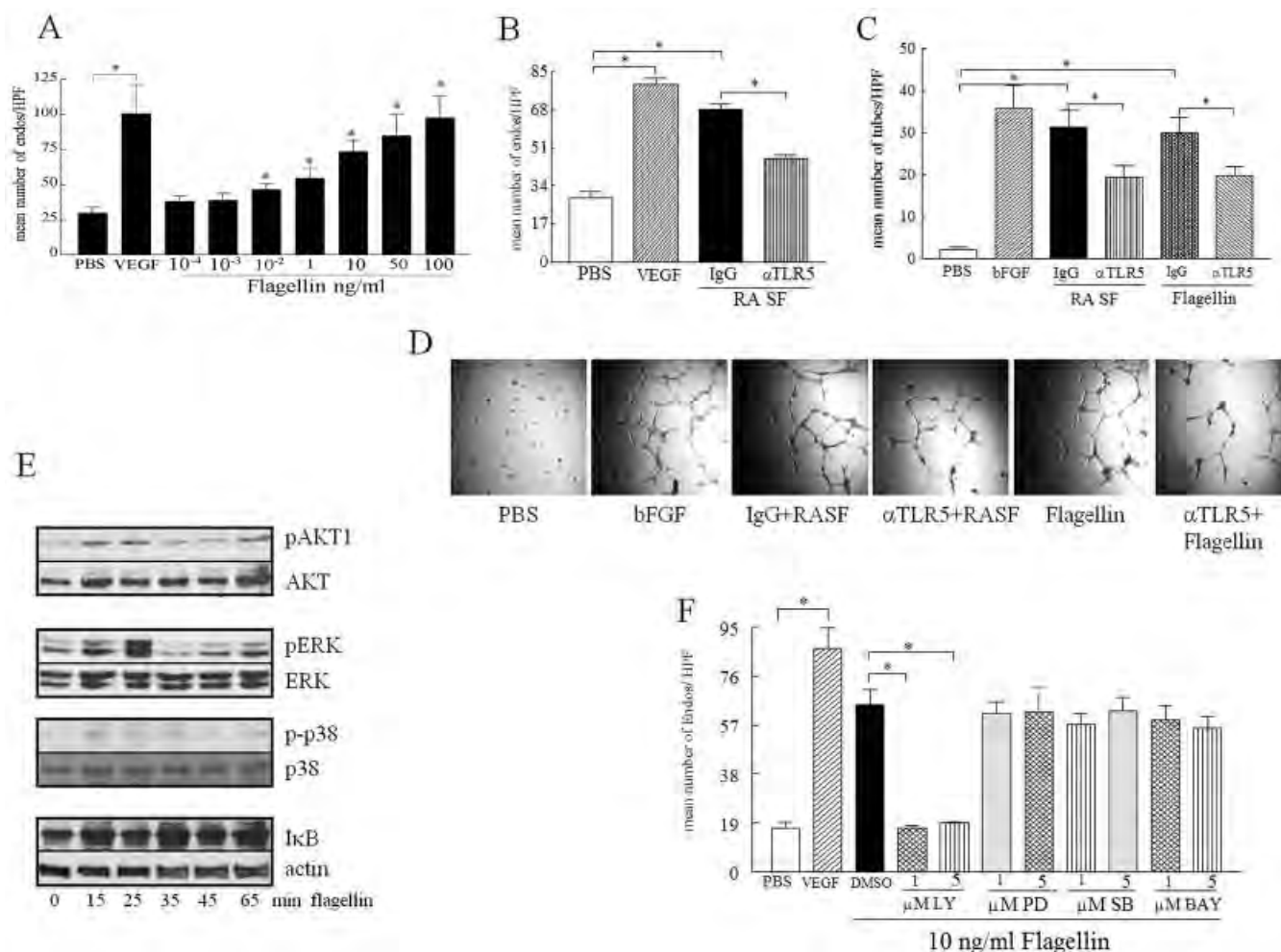To investigate whether flagellin-mediated Th17 cell

Figure 1. Toll-like receptor 5 (TLR-5) ligation promotes endothelial cell (endo) migration and tube formation. **A,** Endothelial cell chemotaxis following exposure to varying concentrations of flagellin (n = 3 experiments). **B** and **C,** Endothelial cell migration (**B**) and tube formation (**C**) in response to rheumatoid arthritis (RA) synovial fluid (SF) (1:20 dilution) (n = 12 RA SF samples) or flagellin (10 ng/ml) (n = 3 experiments). Endothelial cells were preincubated with anti–TLR-5 (10 μg/ml) for 1 hour prior to examining migration and tube formation. **D,** Photomicrographs of representative wells treated with phosphate buffered saline (PBS), basic fibroblast growth factor (bFGF; 20 ng/ml), IgG plus RA SF, anti–TLR-5 plus RA SF, IgG plus flagellin, or anti–TLR-5 plus flagellin. Original magnification × 400. **E,** Western blot analysis of endothelial cell lysates. Cells were stimulated with flagellin (10 ng/ml) for 0–65 minutes, and the cell lysates were probed for phospho–Akt-1, phospho-ERK, phospho-p38, and degradation of IκB (n = 3). **F,** Cells were treated with DMSO or chemical inhibitors of phosphatidylinositol 3-kinase (LY294002 [LY]), ERK (PD98059 [PD]), p38 (SB203580 [SB]), and NF-κB (BAY 11-7082 [BAY]) for 2 hours prior to quantifying endothelial cell chemotaxis in response to flagellin (n = 3 experiments). Bars in **A**–**C** and **F** are the mean ± SEM. ∗ = $P < 0.05$ versus control. hpf = high-power field; VEGF = vascular endothelial growth factor.

polarization is attributable to production of IL-6 and IL-1β by myeloid cells, PBMCs (4×10^6) were maintained in anti-CD3 antibodies (500 ng/ml) and were either left untreated or treated with 10 μg/ml of antibodies to IgG (control), anti–IL-6, anti–IL-1β, or anti–IL-6 plus anti–IL-1β prior to adding flagellin (100 ng/ml) for 5 days. The percentage of Th17 cells or the concentration of IL-17 in supernatants was examined by FACS analysis or ELISA.

Statistical analysis. One-way analysis of variance was used for comparisons among multiple groups, followed by Student's post hoc 2-tailed t-test. Student's paired and un-paired 2-tailed t-tests were used for comparisons between 2 groups. P values less than 0.05 were considered significant.

RESULTS

Activation of the PI3K/Akt-1 pathway contributes to TLR-5–induced angiogenesis. Because TLR-5 expression is elevated in RA compared with normal synovial tissue endothelial cells, we investigated whether ligation of this receptor induces angiogenesis, and
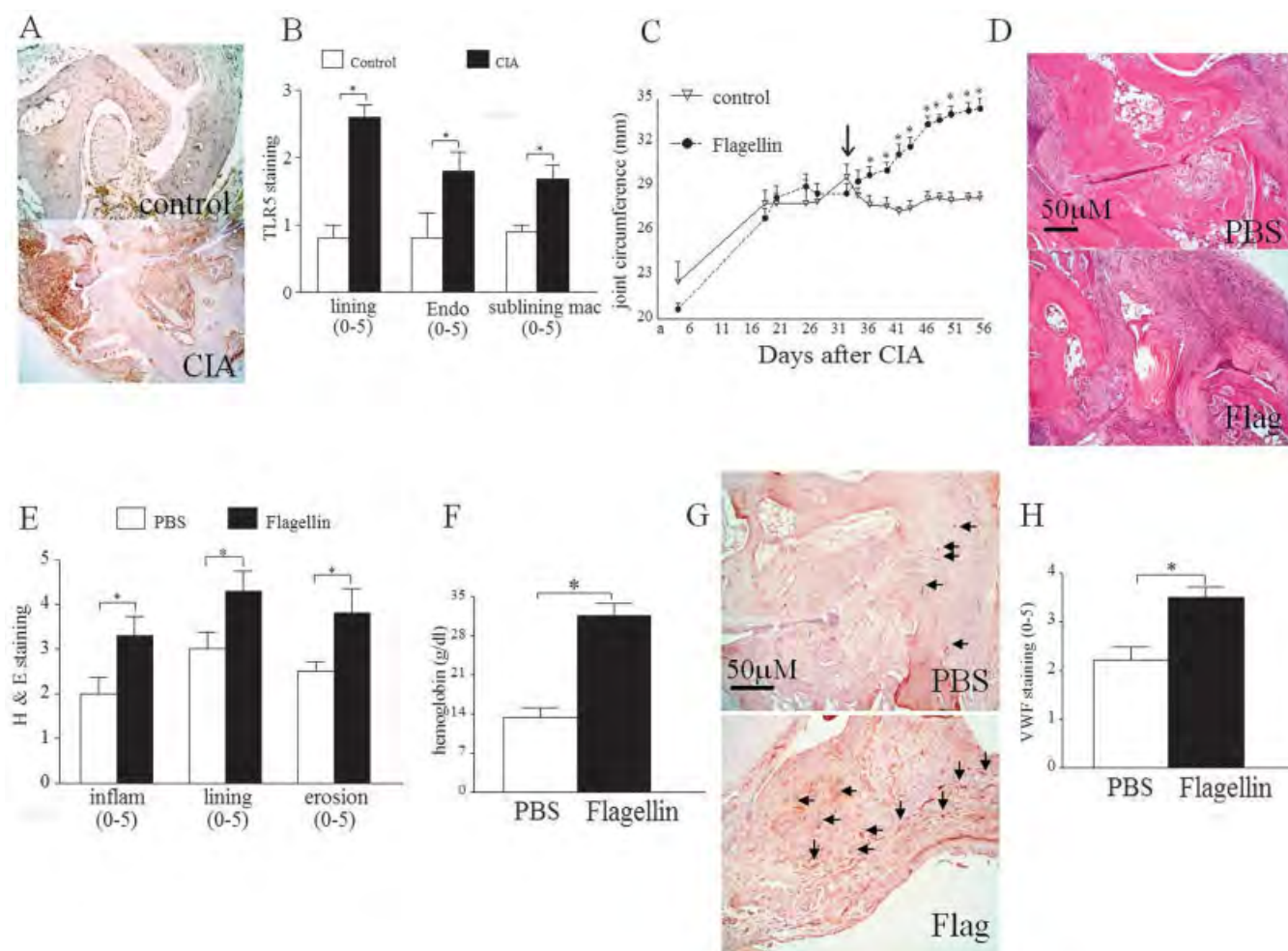
Figure 2. Treatment with TLR-5 agonist after the onset of collagen-induced arthritis (CIA) markedly enhances disease severity and vascularization. **A,** Representative anti–TLR-5 antibody–stained ankle sections from control mice and mice with CIA. Original magnification × 200. **B,** Quantification of TLR-5 staining of synovial tissue lining, endothelial cells (endo), and sublining macrophages (mac), as scored on a 0–5 scale (n = 5–7 experiments). **C,** Changes in joint circumference in mice with CIA (n = 10) that were treated intraperitoneally with PBS (control) or flagellin (Flag; 20 μg). **Arrow** indicates the day on which flagellin treatment was started. **D,** Representative hematoxylin and eosin (H&E)–stained ankle sections from PBS- and flagellin-treated mice with CIA. Original magnification × 200. **E,** Effect of flagellin (administered after the onset of CIA) on ankle joint inflammation (inflam), lining thickness, and bone erosion, as scored on a 0–5 scale (n = 6–8 experiments). **F,** Quantification of hemoglobin levels in ankle sections from mice with CIA that were treated with PBS or flagellin. Ankles were harvested on day 57 after induction of CIA. **G,** Representative von Willebrand factor (vWF) antibody–stained synovial tissue obtained from mice with CIA treated with PBS or flagellin. Tissue was harvested on day 57 after induction of CIA. **Arrows** show vWF-positive staining. Original magnification × 200. **H,** Quantification of vWF staining of endothelial cells, as scored on a 0–5 scale (n = 6–9 experiments). Values are the mean ± SEM. ∗ = $P < 0.05$. See Figure 1 for other definitions.

whether endogenous TLR-5 ligands present in RA SF are involved in this process (10). We observed that when endothelial cells were exposed to varying concentrations of flagellin, flagellin was chemotactic for endothelial cells at concentrations ranging from 0.1 ng/ml to 100 ng/ml ($P < 0.05$) (Figure 1A). Furthermore, incubation of endothelial cells with neutralizing antibody to TLR-5 suppressed RA SF– and flagellin-mediated tube forma-

tion and/or endothelial cell chemotaxis, suggesting that SF TLR-5 endogenous ligands, similar to TLR-5 agonist, can contribute to angiogenesis (Figures 1B–D).

We next demonstrated that although flagellin signaling could phosphorylate the Akt-1 and ERK pathways in endothelial cells as early as 15 minutes after treatment, the p38, NF-κB, and JNK cascades were, surprisingly, not activated (Figure 1E, and results not
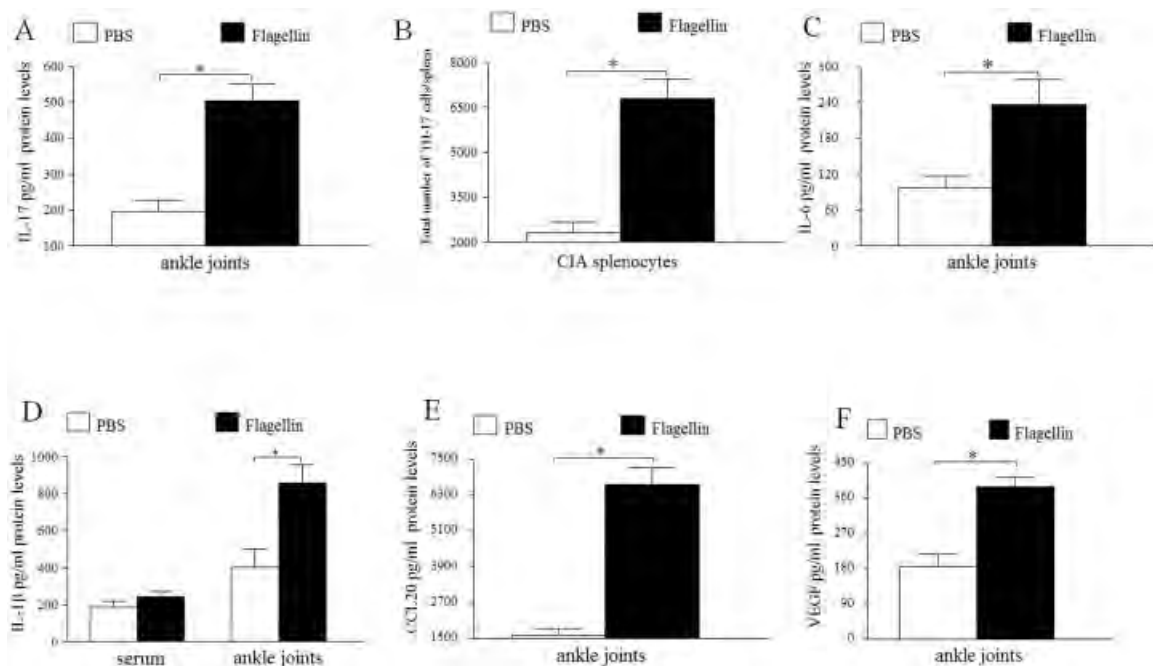
Figure 3. Expression of interleukin-17 (IL-17) and its associated factors is increased in flagellin-treated mice with collagen-induced arthritis (CIA) compared with control mice. **A** and **C–F,** Changes in the protein levels of IL-17 (**A**), IL-6 (**C**), IL-1β (**D**), CCL20 (**E**), and VEGF (**F**) in the joints of mice with CIA treated with PBS or flagellin, as quantified by enzyme-linked immunosorbent assay (n = 6–8 experiments). **B,** Total number of Th17-positive cells per spleen in mice with CIA treated with PBS or flagellin (n = 5), as determined by flow cytometry. Values are the mean ± SEM. ∗ = $P < 0.05$. See Figure 1 for other definitions.

shown). To determine which signaling pathways contribute to flagellin-induced endothelial cell migration, chemical inhibitors at a concentration of 1 μM or 5 μM were used; a concentration of 10 μM was toxic and resulted in cell death, as determined by trypan blue staining (data not shown). Although inhibition of ERK, p38, and NF-κB was ineffective in suppressing flagellin-induced endothelial cell chemotaxis, inhibition of PI3K reduced chemotaxis ($P < 0.05$), starting at a concentration of 1 μM (Figure 1F). These results suggested that ligation of TLR-5 by endogenous RA SF ligands contributed to endothelial cell chemotaxis and tube formation through activation of the PI3K/Akt-1 pathway.

Exacerbation of joint pathology in mice with CIA following treatment with TLR-5 agonist. To further examine the mechanism by which TLR-5 ligation mediates disease, we first determined whether TLR-5 was expressed in the ankle joints of mice with CIA and later sought to determine whether TLR-5 ligation affects CIA pathology. We observed that as in RA, TLR-5 expression was significantly elevated in the lining (70%) and sublining macrophages (50%) and endothelial cells (60%) of mice with CIA compared with controls (Figures 2A and B). We also documented that when mice

with CIA were treated with flagellin, joint inflammation was greatly increased, while the circumference of control ankles remained at a plateau phase (Figure 2C). Consistent with the clinical data, histologic studies demonstrated that in mice with CIA, inflammation (40%), lining thickness (30%), and bone erosion (35%) were significantly elevated by TLR-5 ligation compared with control treatment (Figures 2D and E). Collectively, these results indicated that ligation of TLR-5 promotes the progression of disease in CIA.

Effect of TLR-5 ligation on joint vascularization in mice with CIA. The next experiments were performed to examine whether TLR-5 ligation could contribute to neovascularization in murine models of RA. In agreement with our in vitro findings, we showed that hemoglobin levels were 2-fold higher and vWF staining was 40% higher in flagellin-treated mice compared with control mice (Figures 2F–H). These results suggested that in both RA and CIA, increased joint vascularization is a mechanism by which TLR-5 mediates joint pathology.

Role of TLR-5 ligation in promoting Th17 cell polarization in mice with CIA. Because the severity of disease in mice with CIA was enhanced by flagellin, we evaluated the effect of flagellin treatment on proinflam-
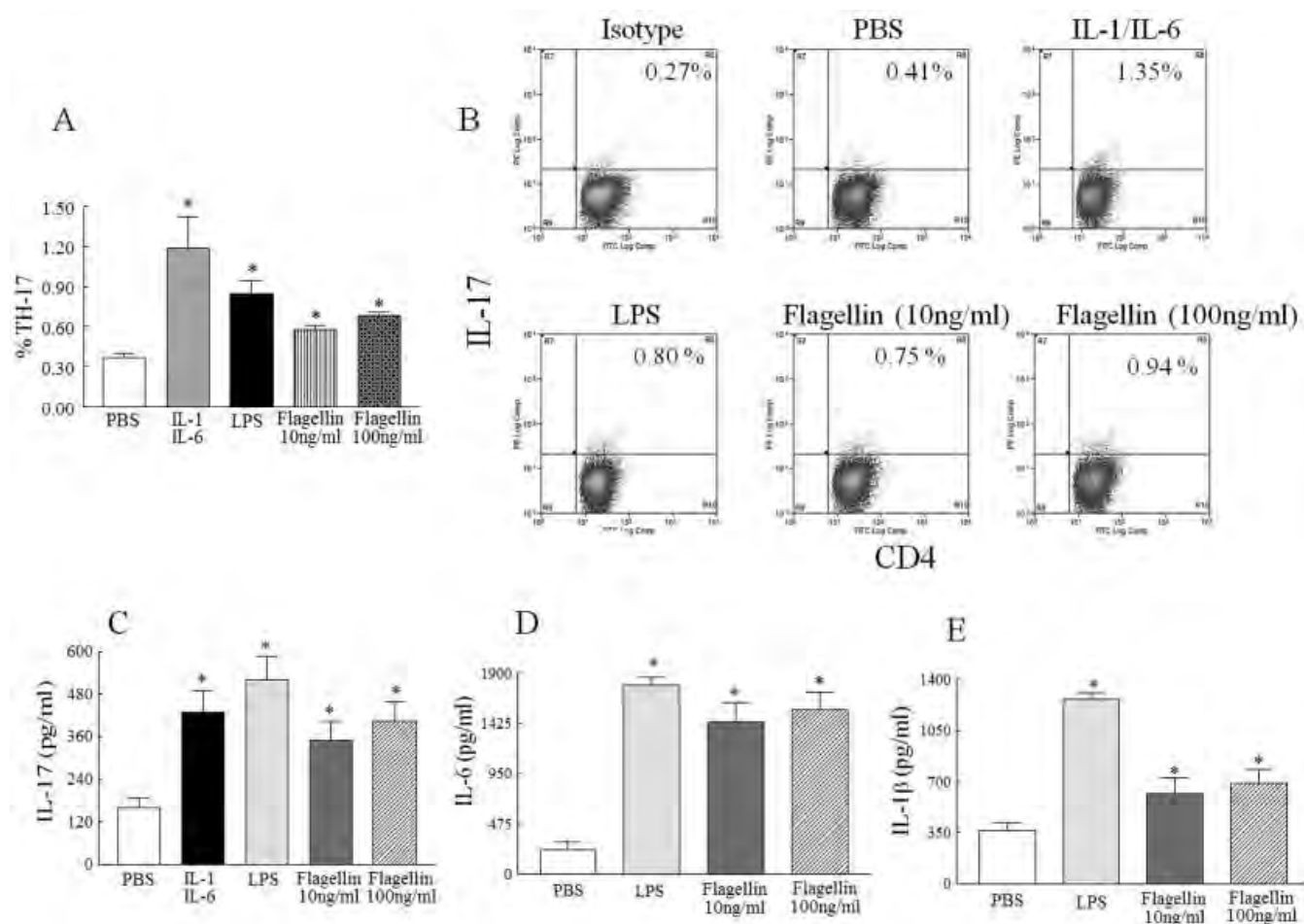
Figure 4. TLR-5 ligation in normal peripheral blood mononuclear cells (PBMCs) drives Th17 cell differentiation. Normal PBMCs were left untreated or were treated with a mixture of interleukin-6 (IL-6), IL-1β, IL-23 (10 ng/ml), transforming growth factor β (TGFβ; 5 ng/ml), lipopolysaccharide (LPS; 100 ng/ml), or flagellin (10 and 100 ng/ml) for 5 days. Cell-conditioned culture medium was harvested, and cells were treated for 4 hours with phorbol myristate acetate (50 ng/ml), ionomycin (750 ng/ml), and brefeldin A (3 μg/ml) to quantify Th17 cells or IL-17 and its related cytokines (n = 3 experiments). **A,** Percentage of Th17 cells in normal PBMCs differentially treated as described above. **B,** Representative flow cytometry histograms of the data shown in **A**. **C–E,** Protein levels of IL-17 (**C**), IL-6 (**D**), and IL-1β (**E**) in normal PBMCs treated as described above, as determined by enzyme-linked immunosorbent assay (n = 3 experiments). Values are mean $\pm$ SEM. $*$ = $P < 0.05$ versus PBS. PE = phycoerythrin; comp = compensation; FITC = fluorescein isothiocyanate (see Figure 1 for other definitions).

matory factors in the joint. Our results demonstrated that IL-17 protein levels in the joint were 2.5-fold higher in flagellin-treated mice compared with control mice (Figure 3A). Based on these observations, we assessed whether the Th17 cell profile was differentially modulated by flagellin treatment in splenocytes from mice with CIA. Consistent with findings in the joints of mice with CIA, we documented that the frequency of Th17 cells in splenocytes was 3-fold lower in control mice compared with flagellin-treated mice (Figure 3B). We also showed that the concentrations of the Th17-promoting cytokines IL-6 and IL-1β were significantly

elevated (50–60%) in the flagellin-treated mice compared with the PBS-treated control mice (Figures 3C and D). Additionally, levels of CCL20 and VEGF, which are Th17 cell–associated factors, were 2–4-fold higher in the flagellin-treated mice compared with the control mice (Figures 3E and F). These results indicated that flagellin treatment promotes Th17 cell polarization through induction of IL-6 and IL-1β expression in the joints of mice with CIA.

Flagellin-induced differentiation of Th17 cells in normal and RA PBMCs. We investigated whether ligation of TLR-5 could modulate human Th17 cell differ-
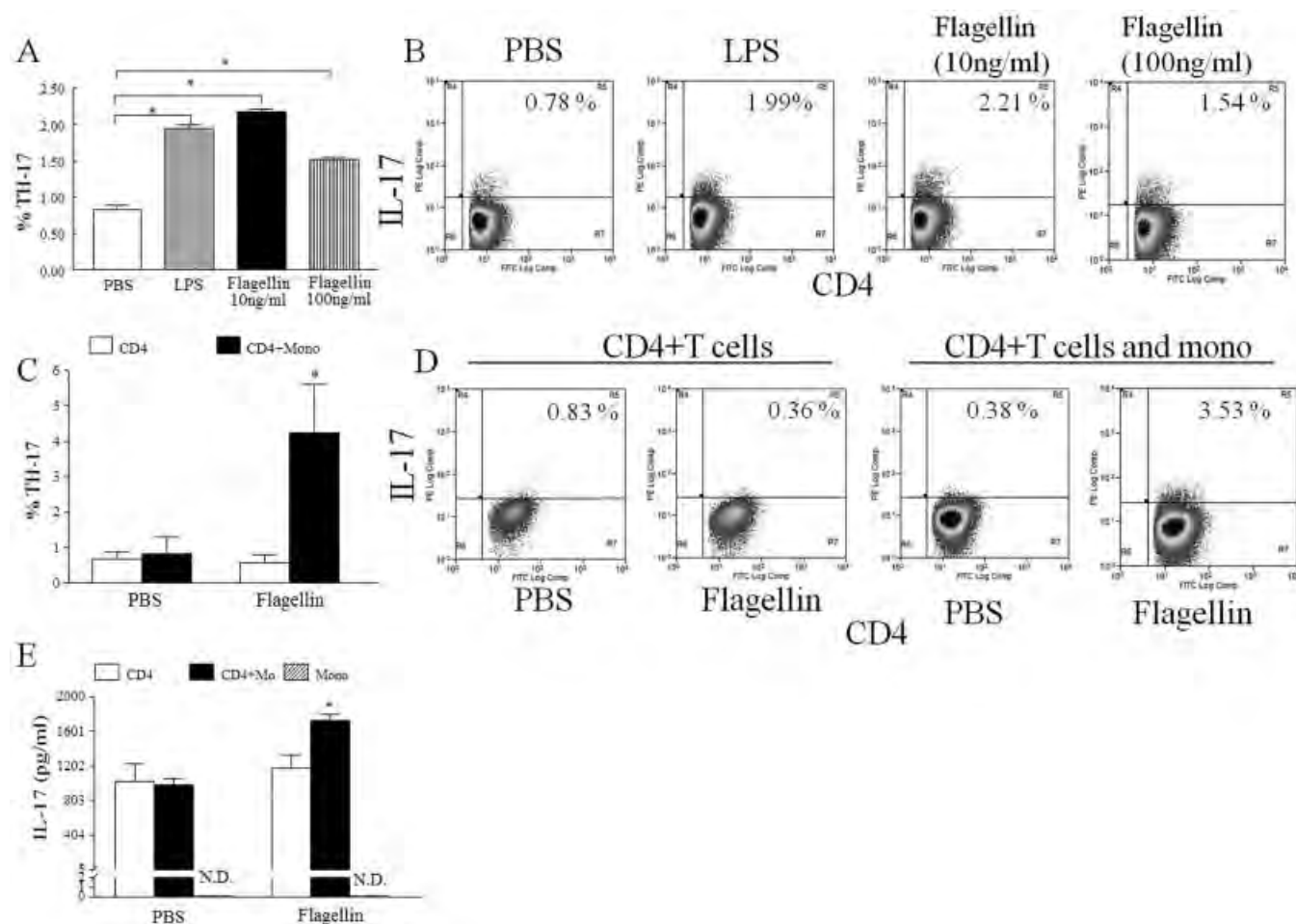
Figure 5. Toll-like receptor 5 (TLR-5) ligation in myeloid cells and interaction between myeloid cells and CD4+ T cells promote Th17 cell differentiation. **A,** Percentage of Th17 cells in PBMCs from patients with rheumatoid arthritis (RA), as determined by fluorescence-activated cell sorting (FACS) analysis. PBMCs from patients with RA were treated as described in Figure 4 for normal cells, with the exception of IL-6 and IL-1β treatment (n = 3 experiments). ∗ = $P < 0.05$. **B,** Representative FACS histograms of the data shown in **A**. **C–E,** Percentage of Th17 cells (**C** and **D**) or concentration of IL-17 (**E**) in supernatants, as determined by FACS analysis (n = 3 experiments) or enzyme-linked immunosorbent assay (n = 6 experiments). RA CD4+ T cells and CD14+ monocytes (Mo, Mono) were negatively selected and cultured either alone or together. Subsequently, cells were treated with phosphate buffered saline (PBS) or flagellin (100 ng/ml) for 5 days. IL-17 protein expression was not determined (ND) in myeloid cells cultured alone (**E**). Bars in **A**, **C**, and **E** are the mean ± SEM. ∗ = $P < 0.05$. See Figure 4 for other definitions.

entiation, or whether this process was specific to mouse cells and/or the inflammatory milieu in CIA. We observed that in PBMCs obtained from healthy donors, ligation of TLR-5 was capable of polarizing Th17 cells (Figures 4A and B) and producing elevated levels of IL-17 as well as IL-6 and IL-1β (Figures 4C–E) in culture supernatants. We documented that the levels of secreted IL-17 were similar to those in cultures with flagellin when normal PBMCs were stimulated with IL-6 plus IL-1β (Figure 4C). As observed in PBMCs from healthy donors, TLR-5 ligation drove Th17 cell devel-

opment in PBMCs from patients with RA (Figures 5A and B).

Next, we sought to determine whether myeloid cell contact with CD4+ cells was essential for Th17 cell differentiation in RA. We observed that flagellin-treated CD4+ T cells alone could not differentiate to Th17 cells, and that interaction with myeloid cells was required for this process (Figures 5C–E). To demonstrate that myeloid cell dependency was attributable to the production of IL-6 and IL-1β by myeloid cells, PBMCs were pretreated with antibodies to IL-6 and IL-1β prior to TLR-5
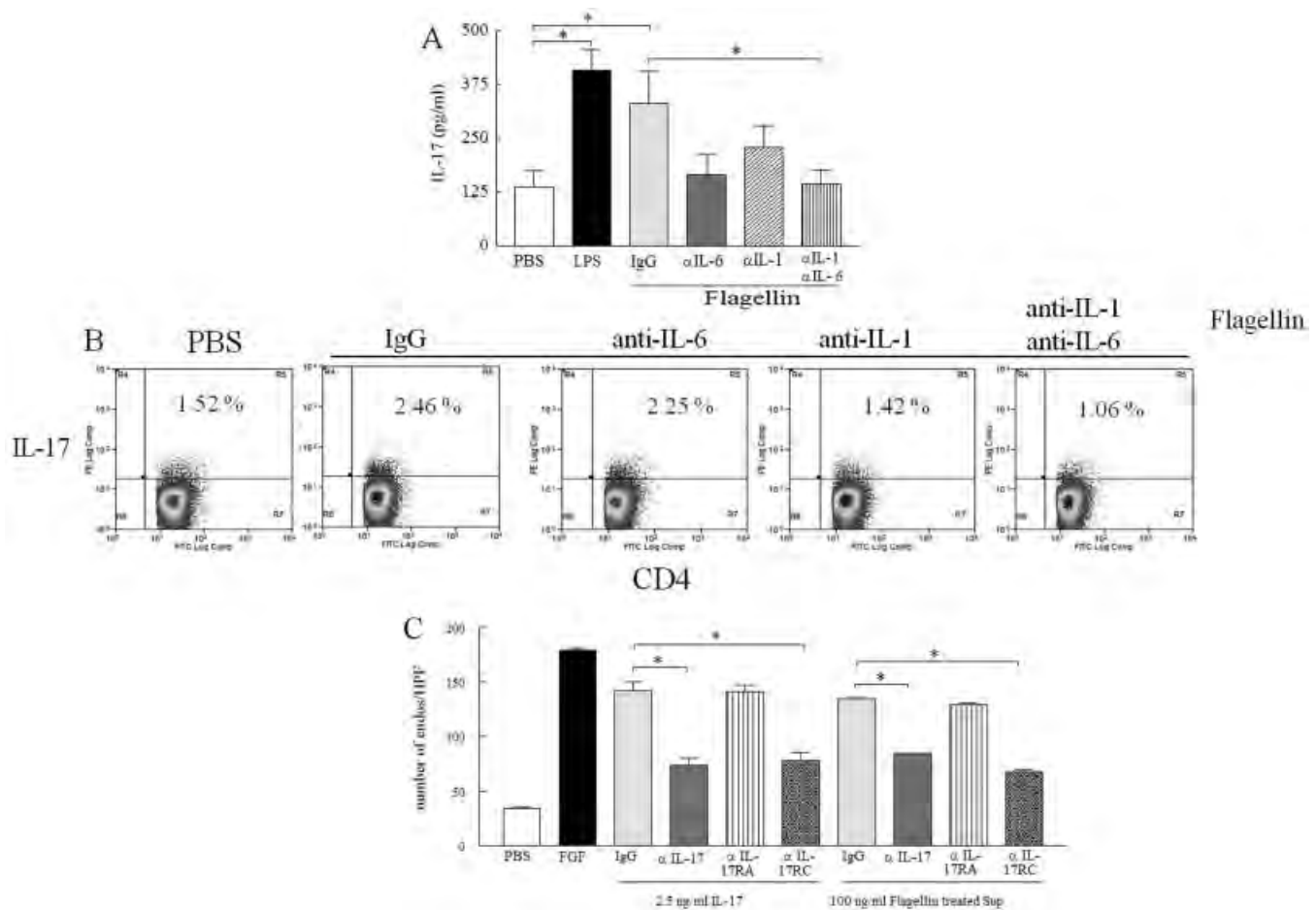
Figure 6. Myeloid cell production of interleukin-6 (IL-6) and IL-1β is responsible for TLR-5–mediated Th17 cell differentiation, and blockade of the IL-17 pathway reduces flagellin-mediated endothelial cell (endo) chemotaxis. Peripheral blood mononuclear cells (PBMCs) were either left untreated or treated with IgG control, anti–IL-6, anti–IL-1β, or anti–IL-6 plus anti–IL-1β antibodies prior to adding flagellin (100 ng/ml) for 5 days. Cells treated with PBS or lipopolysaccharide (LPS; 100 ng/ml) were used as negative and positive controls, respectively. **A** and **B,** Concentration of IL-17 in supernatants (**A**) and percentage of Th17 cells (**B**) as determined by enzyme-linked immunosorbent assay or fluorescence-activated cell sorting analysis (n = 9 experiments). **C,** Migration of endothelial cells treated with IgG (control) or antibodies to IL-17RA or IL-17RC (10 μg/ml) in response to PBMC supernatants (Sup; n = 3 experiments) activated by flagellin (100 ng/ml) or IL-17 (2.5 ng/ml). Endothelial cell chemotaxis in response to flagellin-conditioned medium or IL-17 (2.5 ng/ml) immunoneutralized with anti–IL-17 or IgG control (10 μg/ml) was also determined. Values are the mean ± SEM. ∗ = $P < 0.05$. PE = phycoerythrin; FITC = fluorescein isothiocyanate (see Figure 1 for other definitions).

ligation. Our results clearly demonstrated that myeloid cell production of both IL-6 and IL-1β was critical for flagellin-mediated Th17 cell polarization; thus, neutralization of each factor alone was insufficient in suppressing Th17 cell differentiation driven by flagellin (Figures 6A and B). Collectively, these results suggested that ligation of TLR-5 contributes to myeloid cell production of Th17 cells, promoting cytokines and Th17 cell differentiation in both RA and experimental arthritis.

Role of TLR-5–mediated IL-17 production in endothelial cell migration. In both RA and CIA, TLR-5 ligation strongly induced Th17 cell polarization. We

questioned whether IL-17 detected in flagellin-activated PBMC–conditioned medium is capable of inducing endothelial cell migration. Our data showed that neutralization of IL-17 in culture medium or blockade of IL-17RC in endothelial cells can greatly (40–50%) reduce endothelial cell homing mediated by flagellin-activated conditioned medium (Figure 6C). Our results further demonstrated that endothelial cell chemotaxis promoted by IL-17 was in a range similar to that in supernatants obtained from flagellin-treated cells. Further blockade of IL-17 or endothelial IL-17RC suppressed migration in both treatment groups (IL-17– and

flagellin-treated supernatants) comparably (Figure 6C). Our findings suggested that TLR-5 ligation in myeloid cells that are in close contact with T cells can indirectly result in IL-17–mediated vascularization.

DISCUSSION

Based on the elevated TLR-5 expression detected in RA synovial tissue endothelium (10), we focused on determining the impact of TLR-5 ligation on RA endothelial cell function and on vascularization in CIA. We observed that ligation of TLR-5 by its endogenous ligand present in RA SF contributes to endothelial cell migration and tube formation. Furthermore, treating mice with CIA with the TLR-5 agonist flagellin increased disease severity by enhancing the expression of cytokines that promote Th17 cell development, the frequency of Th17 cells, and joint vascularization. We demonstrate that ligation of TLR-5 in myeloid cells and their production of IL-6 and IL-1β are required for Th17 cell polarization; hence, TLR-5 agonist is incapable of directly promoting IL-17 production by CD4+ or myeloid cells. Last, we document that angiogenesis mediated by TLR-5 ligation is either directly induced through attracting TLR-5–positive endothelial cells or indirectly facilitated via Th17 cell differentiation and IL-17 secretion, thus supporting the novel role of TLR-5 ligation in RA angiogenesis.

It has been shown that TLR-2, TLR-3, TLR-4, TLR-7, and TLR-8 are predominantly expressed in RA synovial tissue lining and sublining myeloid cells (4,5,26,27); therefore, TLR-5 is the only TLR that is uniquely expressed on synovial blood vessels (10). Thus, although TLR-5 ligation can mediate angiogenesis through both direct and indirect mechanisms, other TLRs can induce vascularization only indirectly (15–17).

Our findings are in contrast to those of a previous study in which it was demonstrated that subcutaneously implanted TLR-5–knockdown cells displayed markedly lower neutrophil infiltration due to a reduction in the number of neutrophil chemokines, with no effect on myeloid or endothelial cell recruitment in tumor xenografts (28). We show that inhibition of PI3K/Akt-1 significantly reduces TLR-5–mediated endothelial cell migration, whereas p38, JNK, ERK, or NF-κB signaling is not involved in this process. Interestingly, results from our laboratory and others demonstrate that proangiogenic factors that are highly expressed in RA joints, such as IL-17 (18), CCL21 (19), soluble E selectin (29), macrophage migration inhibitory factor (30), and junctional adhesion molecule C (31), use the PI3K/Akt-1

cascade for inducing endothelial chemotaxis. This suggests that the PI3K/Akt-1 pathway plays a crucial role in RA angiogenesis. Consistent with these findings, reduced cartilage vascularization detected in Akt-1–deficient mice was linked to decreased VEGF expression (32), highlighting the importance of this pathway in modulating the expression of proangiogenic factors as well as neovascularization. In contrast, other investigators have shown that the upstream activator of the JNK pathway, Rac1, is responsible for IL-17–induced tube formation by human dermal endothelial cells (33,34).

The impact of TLR-2 and TLR-4 function in the pathogenesis of RA and experimental models of arthritis is very well studied. In CIA, treatment with TLR-4 antagonist potently reduced clinical and histologic characteristics of arthritis by decreasing expression of IL-1β in joint synovial tissue and articular chondrocytes (35). In contrast, arthritis in TLR-2–deficient mice crossed with IL-1 receptor antagonist–knockout mice was more severe than that in TLR-4–deficient mice crossed with IL-1 receptor antagonist–knockout mice; this was associated with an increase in interferon-γ (IFNγ) expression and a reduction in the number of Treg cells (36). In streptococcal cell wall–induced arthritis, acute joint swelling is dependent on TLR-2, whereas TLR-4 is involved in the chronic and erosive phase of disease by modulating expression of IL-1β, IL-6, and IL-23 and by polarizing Th17 cells (37).

More recently, studies have been performed to determine the role of endosomal TLR-3 and TLR-7 in murine models of RA. Surprisingly, when TLR-3 (38) agonist was used in a model of anti–glucose-6-phosphate isomerase–induced arthritis, joint inflammation was ameliorated as a result of IFNα or IFNγ/IL-12 expression. In contrast, CIA was alleviated in TLR-7–deficient mice due to a reduction in the number of Th17 cells and a trend toward an increased number of Treg cells (39). Other investigators have shown that ligation of TLR-7/8–induced Th17 cell differentiation was driven by IL-23 production in myeloid cells, because the effect was abrogated by IL-23 neutralization (40). However, the pathogenic role of TLR-5 in RA as well as in murine models of RA is completely undefined.

We demonstrate that similar to ligation of TLR-4, ligation of TLR-5 in RA and CIA participates in Th17 cell development by inducing expression of IL-1β and IL-6 in myeloid cells. In contrast to our observations, ligation of TLR-5 in human dendritic cells did not contribute to IL-1β– and IL-6–induced Th17 cell development, while ligation of TLR-2, TLR-3, and TLR-4

facilitated this process (41). This discrepancy may be attributable to several differences in the experiments. In our experiments, monocytes cocultured with CD4+ T cells were activated with 100 ng/ml of flagellin in the presence of 10% medium, and Th17 cells were quantified following 5 days of incubation. In previous studies, dendritic cells were stimulated with 500 ng/ml of flagellin for 24 hours prior to being cocultured with CD4+ T cells in serum-free medium for 7 days (41). Interestingly, in RA macrophages and fibroblasts, expression of TLR-5 is modulated by IL-17 (10), suggesting that there may be cross-regulation between these 2 cascades. Collectively, these results indicate that TLR-5 ligation in myeloid cells and production of Th17 cell–derived factors represent the initial step involved in Th17 cell development. Further, the self-perpetuating feedback regulation detected between the TLR-5 and IL-17 pathways may contribute to perpetuation of arthritis.

It has been shown that the expression of proinflammatory factors is highly elevated in TLR-5–deficient mice, and in a subset of these mice, colitis develops spontaneously through an IL-1β–dependent mechanism. Therefore, TLR-5–deficient mice were not used in this study (42–44). Here, we document that TLR-5 ligation can increase CIA disease severity in part by enhancing joint vascularization through IL-17 and its associated proangiogenic factors. The elevated VEGF levels observed in flagellin-treated mice with CIA is probably attributable to elevated joint expression of IL-17, because TLR-5 ligation has no effect on VEGF transcription (45,46).

Interestingly, TLR-5–, IL-17–, and VEGF-mediated endothelial cell chemotaxis and tube formation are regulated via the PI3K/Akt-1 pathway (18,47,48), suggesting an overlapping mechanism of action. However, recent data demonstrate that similar to TLR-5–mediated angiogenesis, angiogenesis mediated by IL-17 is induced directly via IL-17RC on endothelial cells (18) and/or indirectly through induction of CXCL5 from RA synovial tissue fibroblasts and myeloid cells (49). Therefore, induction of Th17 cells/IL-17 may amplify TLR-5–mediated vascularization in CIA by increasing the CXCL5 production that modulates angiogenesis through activation of NF-κB, which is an IL-17– and TLR-5–nonoverlapping cascade (49). Interestingly, in contrast to findings with TLR-5 and IL-17, endothelial capillary tube formation was suppressed by IL-17F–induced IL-2 and TGFβ production (50).

In conclusion, this study is the first to demonstrate that ligation of TLR-5 in endothelial cells can directly contribute to RA neovascularization, and that TLR-5 agonist can enhance the severity of RA and CIA by elevating Th17 cell polarization and IL-17–mediated angiogenesis.

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Clinical Images: Ancient schwannoma
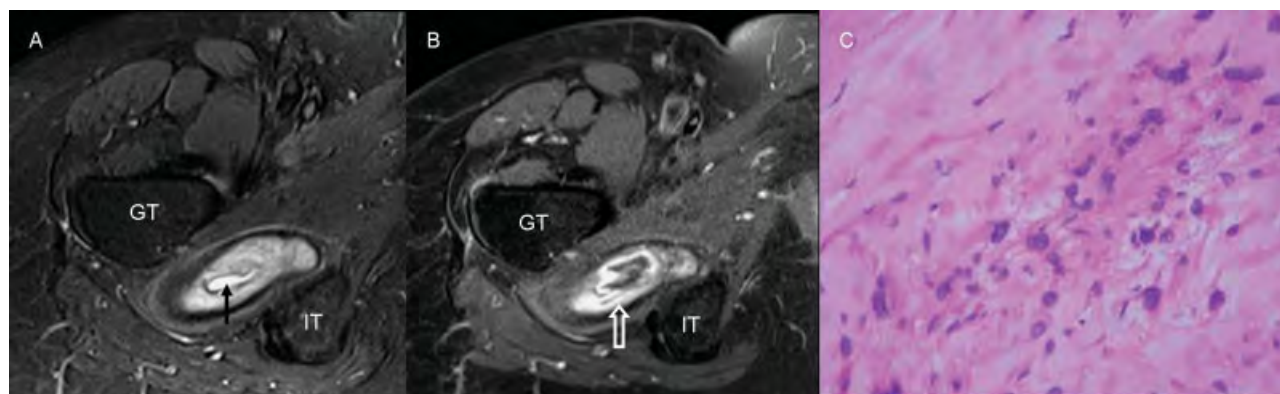
The patient, a 63-year-old woman, presented with pain in the right peritrochanteric region that extended to the gluteus and lasted 3 months. Her movement was not limited, and she had no history of trauma, fever, or constitutional symptoms. During examination, pain was noted on digital compression of the trochanteric area, without signs of inflammation or limitation of active or passive mobilization of the coxofemoral joint. Results of radiographs of the lumbosacral spine and pelvis were normal. Axial STIR sequences (**A**) and T1 sequences with fat suppression after administration of gadolinium (**B**) were obtained on magnetic resonance imaging (MRI) of the right hip. The MRI showed a high signal intensity mass in the soft tissue between the greater trochanter (GT) and the ischial tuberosity (IT). STIR images showed high signal intensity in the central portion of the lesion (**arrow** in **A**) and peripheral and heterogeneous enhancement after administration of gadolinium on T1-weighted sequences (**arrow** in **B**). A biopsy was performed, and histologic analysis (**C**) revealed the presence of a schwannoma with degenerative features (ancient schwannoma), characterized by interstitial hyalinization, degenerative atypia, and cells with large and hyperchromatic nuclei. Schwannomas are benign and encapsulated tumors of the nerve sheath, found most commonly in the extremities, head, and neck (1,2). The term "ancient schwannoma" is used to describe a tumor that has undergone degenerative changes; it is considered a rare condition and is most common in elderly patients (1,2). Although extremely rare, the malignant transformation of ancient schwannoma has been described (3). Ancient schwannoma may be misdiagnosed as other types of tumors based on radiologic findings of cystic degeneration, such as malignant fibrous histiocytoma, malignant peripheral nerve sheath, and synovial sarcoma. Bursae presenting with thick walls and central cystic areas should also be considered in the differential diagnosis. Despite the rarity of these tumors, they should be considered in the differential diagnosis of soft tissue pain, as in the case reported herein.

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INFLAMMATION

A TLR5–TNF positive feedback loop in rheumatoid arthritis

Researchers have reduced the severity of collagen-induced arthritis in mice by blocking Toll-like receptor 5 (TLR5) with a monoclonal antibody. Published in the *Journal of Immunology*, the study also shows that the bacterial TLR5 agonist flagellin can convert osteoclast precursor cells into fully mature osteoclasts and stimulate chemotaxis of monocytes from patients with rheumatoid arthritis (RA).

Osteoclast differentiation in patients with RA is thought to involve TLR-activated proinflammatory cytokines such as TNF. Endogenous ligands for TLR2 and TLR4 are part of the inflammatory cascade in RA synovium, and TLR4 is involved in the pathogenesis of experimental arthritis in mice. Furthermore, Shiva Shahrara, corrresponding author of the study, says "TLR5 is highly elevated in RA compared to normal synovial tissue myeloid and endothelial cells." However, until now, a role for TLR5 in the pathogenesis of RA was unclear.
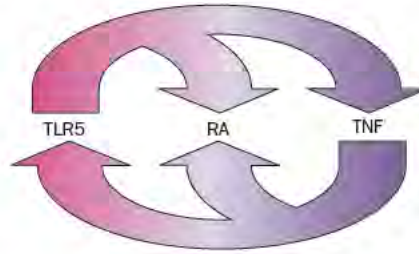
The researchers isolated peripheral blood mononuclear cells from patients with RA treated with DMARDs or with TNF inhibitors plus or minus DMARDs.

Flagellin treatment of monocytes *in vitro* had an NF-κB-dependent, dose-dependent, positive effect on chemotaxis, and anti-TLR5 antibody treatment inhibited chemotaxis of these cells.

Monocytes from patients treated with TNF inhibitors had lower TLR5 expression than monocytes from patients who were treated with DMARDs without TNF inhibitors, suggesting that TNF

and TLR5 pathways synergise to effect RA pathogenesis. Indeed, Shahrara and colleagues showed that blocking either TNF or TLR5 signalling in mice prevents flagellin-induced osteoclastogenesis.

Timothy Radstake, an independent expert from the University Medical Center Utrecht, The Netherlands, warns "As there is no endogenous ligand for TLR5 known so far, the true value for arthritis has to be investigated further," but he says "this new study ignites the search".

Shahrara responds "We believe TLR5 is the bridge that interconnects formation of new blood vessels with maturation of joint osteoclasts; thereby accelerating the bone destruction process in RA."

Nicholas J. Bernard

Ligation of TLR5 Promotes Myeloid Cell Infiltration and Differentiation into Mature Osteoclasts in Rheumatoid Arthritis and Experimental Arthritis

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Michael V. Volin,[†] M. Asif Amin,[‡] Suncica Volkov,* Ellen M. Gravallese,[§]
Shiva Arami,* William Swedler,* Nancy E. Lane,[¶] Anjali Mehta,* Nadera Sweiss,* and
Shiva Shahrara*

Our aim was to examine the impact of TLR5 ligation in rheumatoid arthritis (RA) and experimental arthritis pathology. Studies were conducted to investigate the role of TLR5 ligation on RA and mouse myeloid cell chemotaxis or osteoclast formation, and in addition, to uncover the significance of TNF-α function in TLR5-mediated pathogenesis. Next, the in vivo mechanism of action was determined in collagen-induced arthritis (CIA) and local joint TLR5 ligation models. Last, to evaluate the importance of TLR5 function in RA, we used anti-TLR5 Ab therapy in CIA mice. We show that TLR5 agonist, flagellin, can promote monocyte infiltration and osteoclast maturation directly through myeloid TLR5 ligation and indirectly via TNF-α production from RA and mouse cells. These two identified TLR5 functions are potentiated by TNF-α, because inhibition of both pathways can more strongly impair RA synovial fluid driven monocyte migration and osteoclast differentiation compared with each factor alone. In preclinical studies, flagellin postonset treatment in CIA and local TLR5 ligation in vivo provoke homing and osteoclastic development of myeloid cells, which are associated with the TNF-α cascade. Conversely, CIA joint inflammation and bone erosion are alleviated when TLR5 function is blocked. We found that TLR5 and TNF-α pathways are interconnected, because TNF-α is produced by TLR5 ligation in RA myeloid cells, and anti TNF-α therapy can markedly suppress TLR5 expression in RA monocytes. Our novel findings demonstrate that a direct and an indirect mechanism are involved in TLR5-driven RA inflammation and bone destruction. *The Journal of Immunology*, 2014, 193: 3902 3913.

R heumatoid arthritis (RA) is a chronic autoimmune disorder in which the numbers of monocyte derived macrophages are greater than in normal (NL) joints and are well correlated with radiological damage, joint pain, and inflammation (1, 2). Yet, the mechanism that derives RA myeloid cell trafficking and further facilitates their maturation to osteoclasts is incompletely understood.

Osteoclasts are multinucleated bone resorbing cells differentiated from the myeloid lineage found in RA peripheral blood (PB) and synovial tissue (ST) (3 5). In RA, dominance of proin flammatory factors such as TNF α, IL 1β, IL 6, and IL 17 can promote osteoclast maturation through enhancing myeloid re ceptor activator for NF κB (RANK) expression, as well as RANK ligand (RANKL) production from RA ST fibroblasts and T cells (4, 6 9). Interestingly, ligation of TNF α to myeloid TNFR1 and TNFR2 can directly facilitate osteoclast differentiation through a mechanism that is independent of RANK/RANKL cascade (10, 11). Confirming this notion, others have shown that RA synovial fluid (SF) macrophages can transform into mature osteoclasts in the presence of M CSF in combination with RANKL or TNF α/ IL 1β, suggesting that proinflammatory factors such as TNF α/ IL 1β can replace the function of RANKL (12), whereas TNF α and IL 17 are known to be responsible for joint myeloid cell re tention directly through myeloid cell ligation and indirectly via induction of monocyte chemokines (13 15).

TLRs belong to the family of pattern recognition receptors, and TLR2 and TLR4 are abundantly expressed in RA PB monocytes, RA SF, and ST macrophages (16 19). Identification of TLR2 and TLR4 endogenous ligands in RA synovium has triggered an in terest in discovering the role of these receptors in the RA patho genesis (20). Hence the impact of TLR2 and TLR4 ligation has been extensively studied on bone degradation, primarily in mouse bone marrow cells (21 23) with a few studies performed in NL human myeloid cells (24). Despite these comprehensive in vitro investigations, the obtained results are controversial and the effect of TLR4 ligation on osteoclast differentiation is greatly dependent on the treatment time point, the cell type used, and the concentration of reagents used (21, 24, 25). However, the in vivo studies consis tently support the significance of TLR4 activation in experimental

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Abbreviations used in this article: CIA, collagen-induced arthritis; CTR, calcitonin receptor; CTSK, cathepsin K; DMARD, disease-modifying antirheumatic drug; HSP, heat shock protein; i.a., intra-articular(ly); NL, normal; PB, peripheral blood; RA, rheumatoid arthritis; RANK, receptor activator for NF-κB; RANKL, RANK ligand; SF, synovial fluid; ST, synovial tissue; TRAP, tartrate-resistant acid phosphatase.

arthritis bone loss (26 28). Unlike TLR2 and TLR4, the role of TLR5 in RA and murine models of RA is undefined.

In our recent article, we uncovered for the first time, to our knowledge, that the TLR5 expression is markedly accentuated in RA compared with NL ST and PB myeloid cells (29). We also found that ligation of myeloid TLR5 to potential endogenous ligands in the RA joint can modulate SF TNF α transcription (29). Notably, expression of myeloid TLR5 closely correlates with RA disease activity and TNF α levels (29), suggesting that ligation of TLR5 in RA myeloid cells contributes to disease progression. Therefore, the significance of the TLR5 cascade was investigated in myeloid cell function using RA PB myeloid cells and mouse PB and bone marrow cells, as well as in acute and chronic experimental arthritis models.

In this study, we demonstrate that the TLR5 agonist, flagellin, can dose dependently promote monocyte migration and osteoclast maturation through its direct effect on myeloid cell function and indirectly via TNF α production from RA and mouse myeloid cells or collagen induced arthritis (CIA) ankle joints. Conversely, anti TLR5 Ab therapy attenuates CIA joint myeloid cell homing and bone erosion. Consistent with our findings in RA, flagellin treatment can strongly transform mouse bone marrow progenitor cells into mature osteoclasts through a TNF α dependent and IFN β independent mechanism. In conclusion, a strong positive feedback regulation exists between TLR5 and TNF α pathways in attracting the circulating monocytes and further remodeling the newly recruited cells into mature osteoclasts; therefore, disruption of TLR5 ligation can dysregulate both functions in preclinical arthritis models.

Materials and Methods

Monocyte chemotaxis

Experiments were performed to determine the effect of flagellin on monocyte chemotaxis. Mononuclear cells were isolated by Histopaque (GE Healthcare Bio Sciences, Pittsburgh, PA) gradient centrifugation, and monocytes were isolated from NL or RA PB using negative selection kit (Stem Cell Technology, Vancouver, BC) according to the manufacturer's instruction (30, 31). Chemotaxis was performed in a Boyden chamber (Neuro Probe, Gaithersburg, MD) using NL monocytes for 2 h with varying concentrations (0.001 100 ng/ml) of flagellin (Ultrapure; endotoxin level <50 endotoxin units/mg; Invivogen, San Diego, CA), fMLF (f; 10 nM) was used as positive control, and PBS was used as negative control (14, 15). Cell culture media, FBS, culture plates, and all reagents used were tested for endotoxin contamination.

To demonstrate that RA SF mediated monocyte trafficking involves TLR5 ligation, we incubated cells with anti TLR5 (10 μg/ml; Invivogen) or IgG Abs for 1 h before performing monocyte chemotaxis in response to eight different RA SFs (20% dilution). To show that TLR5 and TNF α pathways are interconnected in facilitating monocyte migration, we incubated RA SFs (20%) with IgG or anti TNF α (10 μg/ml; R&D Systems), and monocytes were immunoneutralized by either anti TLR5 or IgG Abs (10 μg/ml) before performing monocyte chemotaxis.

To examine the signaling pathways associated with flagellin induced monocyte chemotaxis, we treated monocytes with DMSO or 1 and 5 μM inhibitors to PI3K (LY294002), ERK (PD98059), p38 (SB203580), JNK (SP600125), and NF κB (Bay 11 7085) (EMD Millipore, Billerica, MA) for 1 h. Subsequently, monocyte chemotaxis was performed in response to 100 ng/ml flagellin.

To document that flagellin and TNF α synergistically contribute to monocyte chemotaxis, we examined monocyte migration in response to various concentrations of flagellin (0.1 and 10 ng/ml) or TNF α (0.1 and 5 ng/ml) alone or in combination.

Flagellin signaling in monocytes

NL monocytes were untreated or treated with flagellin (100 ng/ml) for 15 65 min. Cell lysates were examined by Western blot analysis (14, 15). Blots were probed with p AKT1, p ERK, p p38, p JNK, p paxillin, and p FAK (1:1000 dilution; Cell Signaling) and IκB Abs (1:3000 dilution; Santa Cruz) or probed with AKT, ERK, p38, or actin Abs (1:3000 dilution; Cell Signaling or Sigma).

RA patient population

These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. RA patients were diagnosed according to the 1987 revised criteria from the American College of Rheumatology (32). PB was obtained from 68 patients, 64 women and 4 men (mean age 48.6 ± 14.6 y). At the time of evaluation, patients were either on disease modifying antirheumatic drugs (DMARDs; n 34, 3 men and 31 women, mean age 51.6 ± 16.2) or treatment with anti TNF therapy (n 34, 1 man and 33 women, mean age 45.7 ± 12.4). Treatment with DMARDs (n 34) consisted of DMARDs alone (methotrexate, leflunomide, sulfasalazine, azathioprine, hydroxy chloroquine, or minocycline; n 27), of which two were on hydroxy chloroquine only, or treatment with DMARD plus prednisone (n 7). Patients treated with anti TNF therapy (n 34) were either on anti TNF therapy alone (n 7), anti TNF plus prednisone (n 1), anti TNF therapy plus DMARD (n 21), or anti TNF with DMARD and prednisone (n 5).

Quantification of tartrate resistant acid phosphatase⁺ cells in in vitro and in vivo experiments

To generate mature osteoclasts, we cultured NL or RA PBMCs or monocytes in 0% RPMI media and allowed them to attach for 1 h. Thereafter, cells were cultured in 10% α MEM and were either untreated (negative control) or treated with 20 ng/ml human M CSF and RANKL (positive control; ProSpec, Brunswick, NJ) for 14 21 d with the reagents and the culture media replenished every 3 d. The ability of test reagents to differentiate human precursor cells to fully mature osteoclasts was examined in the suboptimal culture conditions that consisted of 10 ng/ml human M CSF and RANKL. To quantify osteoclast formation, we performed tartrate resistant acid phosphatase (TRAP) staining using Acid Phosphatase Leukocyte Kit (Sigma Aldrich) in cells or mice ankles according to the manufacturer's instructions. In vitro experiments were performed in triplicates, and the total number of osteoclasts was determined by counting TRAP⁺ multinuclear (more than four nuclei) cells in each well.

To examine the impact of TLR5 ligation on osteoclastogenesis, we exposed NL and RA PBMCs to varying concentrations of flagellin (0.001 100 ng/ml) in suboptimal culture conditions (10 ng/ml human M CSF and RANKL) before TRAP staining.

To determine whether T cell function plays a critical role in flagellin mediated osteoclastogenesis, we cultured negatively selected RA myeloid cells in suboptimal conditions in the presence or absence of flagellin (10 ng/ml) before TRAP staining.

To show that flagellin mediated osteoclast maturation is due to TLR5 ligation and TNF α induction, we incubated RA PBMCs cultured in suboptimal conditions with 10 μg/ml IgG, anti TNF α, or anti TLR5 before flagellin (10 ng/ml) treatment and followed by TRAP staining.

RA PBMCs cultured in suboptimal conditions were either untreated or treated with TNF α (1 ng/ml), flagellin (1 ng/ml) alone, or in combination before TRAP staining, to demonstrate whether TLR5 and TNF α pathways synergize in promoting osteoclast differentiation.

To determine that in RA joint, TLR5 and TNF α mediated osteoclastogenesis are interconnected, we immunoneutralized RA PBMCs cultured in suboptimal conditions by anti TLR5 or IgG control (10 μg/ml), and cells were then incubated with 2% RA SF plus IgG or 2% RA SF plus anti TNF α (10 μg/ml) before TRAP staining.

To generate mature osteoclasts from mouse PB, we isolated mononuclear cells by Histopaque (GE Healthcare Bio Sciences) gradient centrifugation, and mouse monocytes were isolated by negative selection kit (catalog no. 19701A; Stem Cell Technology) according to the manufacturer's instructions. Thereafter, cells were cultured in 10% α MEM and were treated with 20 ng/ml mouse M CSF and RANKL (suboptimal condition) (R&D Systems) with or without flagellin (10 ng/ml) for 14 21 d. The reagents and culture media were replenished every 3 d. Mouse PB monocytes cultured in 10% α MEM alone were considered as negative control, and cells cultured in presence of 40 ng/ml M CSF and 40 ng/ml RANKL served as the positive control.

To examine the role of TLR5 ligation in mouse bone marrow derived osteoclasts, we isolated bone marrow cells from C57/BL6 mice femur and tibia. Nonadherent mouse bone marrow cells (5 × 10⁵ cells/96 wells) from 1 h culture were incubated for 4 d in 10% α MEM supplemented with 10 ng/ml mouse M CSF plus 25 ng/ml mouse RANKL (suboptimal condition). After 4 d culture, cells were untreated or stimulated with flagellin (10 ng/ml) plus IgG (10 μg/ml), flagellin (10 ng/ml) plus anti TLR5 (10 μg/ml; Invivogen), or flagellin (10 ng/ml) plus anti TNF α (10 μg/ml) for 3 additional days before TRAP staining. Nonadherent mouse bone marrow cells cultured in 10% α MEM alone were considered as negative control, and cells cultured in the presence of

mouse 10 ng/ml M CSF and 100 ng/ml RANKL were regarded as positive control.

To determine that TLR5 ligation contributes to bone erosion in acute and chronic models of arthritis, we TRAP stained ankles ectopically treated with flagellin or vehicle, as well as ankle joints harvested from CIA post onset treatment of flagellin or PBS. To document that blockade of TLR5 ligation can impair osteoclast differentiation, we performed TRAP staining on CIA ankle joints harvested from IgG or anti TLR5 Ab therapy. Number of osteoclasts stained in mouse ankles was quantified by counting the number of TRAP$^+$ cells in each section (33).

FACS analysis

Cells were blocked with 50% human serum in 0.5% BSA to determine the percentage of TLR5$^+$ T cells in NL and RA PBMCs. Subsequently, cells were stained with PE conjugated anti TLR5 (Imgenex, San Diego, CA) and FITC labeled anti CD3 (BD Pharmingen, Franklin Lakes, NJ) or IgG Abs (BD Pharmingen).

Real time RT PCR

Total cellular RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA), and relative gene expression was determined by real time RT PCR using the $\Delta\Delta C_t$ method (29 31). To determine the mechanism by which TLR5 ligation promotes osteoclast formation, we cultured RA PBMCs for 7 d at suboptimal conditions (10 ng/ml M CSF and RANKL). Thereafter, cells cultured in α MEM in the absence of M CSF and RANKL were untreated or stimulated with flagellin (100 ng/ml) for 6 h, and the expression of RANK, RANKL, TNF α, and IFN β was quantified. TNF α and IFN β real time RT PCR was performed on mouse osteoclasts precursor cells from day 4 cultured in 10 ng/ml mouse M CSF plus 25 ng/ml

mouse RANKL treated with or without flagellin (100 ng/ml) for 6, 24, 48, and 72 h. In addition, transcription of calcitonin receptor (CTR), cathepsin K (CTSK), RANKL, and IFN β was determined in CIA ankles treated with flagellin (20 μg) or PBS. TLR5 expression was quantified in RA monocytes treated with DMARDs or anti TNF α with or without DMARDs.

Cytokine quantification

Mouse TNF α protein concentration was determined by ELISA (R&D Systems) according to the manufacturer's instructions in CIA mouse ankles treated with PBS or flagellin (20 μg), as well as in day 4 mouse bone marrow precursor cells untreated (PBS) or treated with flagellin (100 ng/ml) plus IgG (10 μg/ml) versus flagellin (100 ng/ml) plus anti TLR5 (10 μg/ml) for 24 h. Joint IL 6 and CCL2 protein levels were determined in CIA mice treated with IgG or anti TLR5 Ab.

Study protocol for animal models

Eight week old DBA/1 mice were immunized with collagen type II (Chondrex, Redmond, WA) on days 0 and 21 (34, 35). Flagellin (20 μg, n 10; Invivogen) or PBS (n 10) was injected i.p. on day 33 after CIA induction, mice were sacrificed on day 57, and experiments were repeated twice. Ankle circumference was determined by Caliper using the following formula: Circumference 2B $\times$ ($\sqrt{[a^2 + b^2/2]}$), where a and b represent the diameters (34 36). In a different experiment, C57BL/6 mice were injected intra articularly (i.a.) on day 0 with 20 μg flagellin or PBS, mice were sacrificed on day 10, and ankle circumference was determined by Caliper (34 36). To validate that the loss of TLR5 function can critically impact joint inflammation and bone erosion, after CIA induction (34, 35), we treated mice i.p. with IgG or
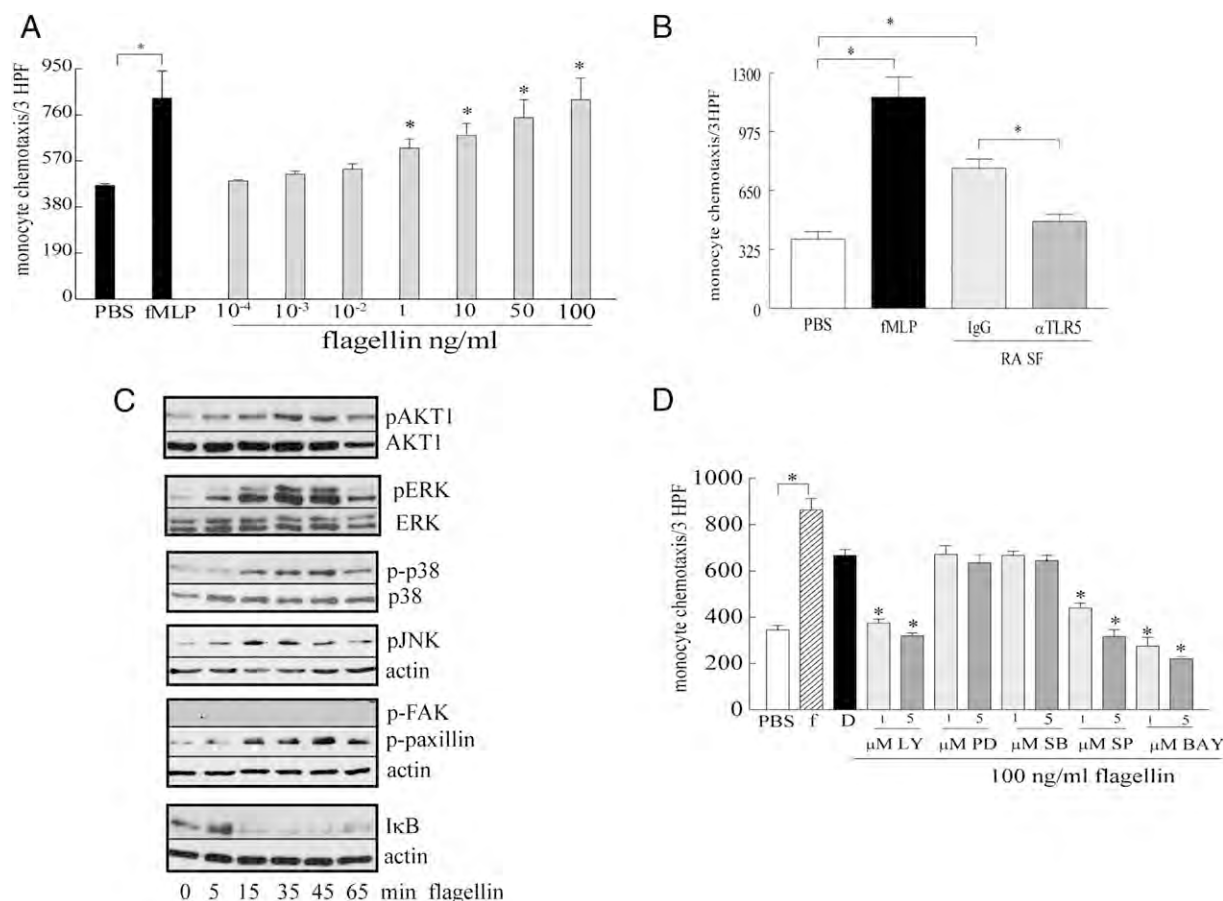

FIGURE 1. TLR5 ligation promotes monocyte migration through activation of AKT1/PI3K, JNK, and NF κB pathways. (**A**) Flagellin monocyte chemotaxis was performed in a Boyden chemotaxis chamber with varying concentration (0.001 100 ng/ml), n 3. (**B**) Monocytes were incubated with anti TLR5 Ab (10 μg/ml) or control IgG for 1 h; thereafter chemotaxis was performed in response to 20% RA SF (n 8). (**C**) Monocytes were stimulated with 100 ng/ml flagellin for 0 65 min, and the cell lysates were probed for p AKT1, p ERK, p p38, p JNK, p FAK, p paxillin, and degradation of IκB (n 3). (**D**) Cells were preincubated with DMSO (D) or 1 and 5 μM inhibitors to PI3K (LY294002), ERK (PD98059), p38 (SB203580), JNK (SP600125), and NF κB (Bay 11 7085) for 1 h. Subsequently, monocyte chemotaxis was performed in response to 100 ng/ml flagellin for 2 h (n 3). For all experiments, PBS and fMLF (f) served as negative and positive controls. Values demonstrate mean $\pm$ SE. *$p < 0.05$.

monoclonal rat anti mouse TLR5 Ab (100 μg/injection; Invivogen) on days 23, 27, 30, 34, 37, 41, 44, and 48. Animals were sacrificed on day 49 after induction.

Immunohistochemistry

Mouse ankles were decalcified, formalin fixed, paraffin embedded, and sectioned. In brief, slides were deparaffinized in xylene, and Ags were unmasked by incubating slides in Proteinase K digestion buffer (Dako, Carpinteria, CA). Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako). CIA ankles were stained with F480 (1:100 dilution; Serotec), iNOS (1:200 dilution; Santa Cruz), or control IgG Abs (Beckman Coulter). Joint myeloid cell or M1 macrophage staining was scored on a 0 5 scale by two blinded observers.

Statistical analysis

One way ANOVA was used for comparison among multiple groups followed by post hoc two tailed Student t test. The data were also an alyzed using two tailed Student t test for paired or unpaired compar isons between two groups. The p values < 0.05 were considered significant.

Results

Activation of PI3K/AKT1, JNK, and NF κB pathways contributes to TLR5 induced monocyte chemotaxis

Because TLR5^{+} monocyte derived macrophages are elevated in RA compared with NL ST, we asked whether circulating mono cytes can migrate into the RA joint where TLR5 endogenous ligands are expressed (29). We found that flagellin is chemotactic for monocytes at concentrations ranging from 1 to 100 ng/ml (Fig. 1A). Further blockade of myeloid TLR5 suppressed RA SF mediated monocyte chemotaxis, suggesting that the potential TLR5 endogenous ligands can contribute to joint monocyte homing (Fig. 1B). We next demonstrate that monocytes stimulated with flagellin phosphorylate AKT1, ERK, p38, JNK, paxillin, and degrade IκB pathways; in contrast, FAK was not activated by TLR5 ligation (Fig. 1C). Interestingly, although inhibition of ERK and p38 was ineffective, suppression of PI3K, JNK, and NF κB pathways markedly reduced flagellin mediated chemotaxis start ing at 1 μM (Fig. 1D). These results suggest that ligation of TLR5
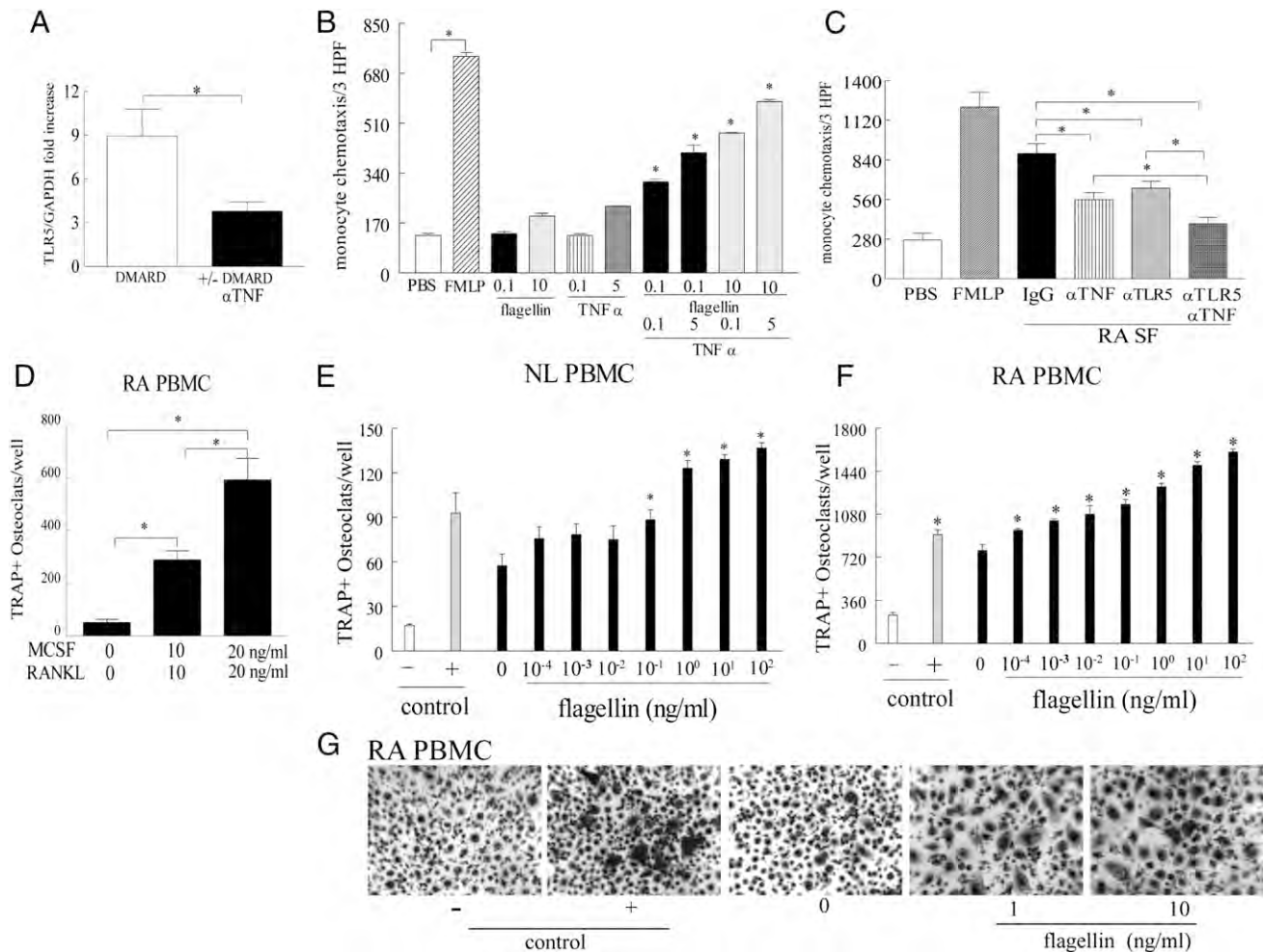
FIGURE 2. TLR5 mediated monocyte trafficking is interconnected to TNF α pathway, and ligation of myeloid TLR5 is a strong promoter of RA os teoclast differentiation. (**A**) Expression of TLR5 was quantified in RA monocytes treated with DMARDs (n 34) or with anti TNF α in the presence or absence of DMARDs (n 34). (**B**) Monocyte migration was examined in response to various concentrations of flagellin (0.1 and 10 ng/ml) or TNF α (0.1 and 5 ng/ml) alone or in combination. The combined doses were compared with the same doses treated alone (n 3). (**C**) Twenty percent RA SFs were incubated with 10 μg/ml IgG or anti TNF α, and monocytes were either immunoneutralized by 10 μg/ml anti TLR5 or IgG control before performing chemotaxis (n 7). (**D**) RA PBMCs were differentiated to fully mature osteoclasts in the presence 20 ng/ml M CSF and RANKL, whereas suboptimal conditions consisted of 10 ng/ml M CSF and RANKL (n 4) (**E**) NL (n 3) and RA PBMCs (n 4) (**F**) were exposed to varying concentrations of flagellin (0.001 100 ng/ml) in the presence of 10 ng/ml human M CSF and RANKL (suboptimal condition) before TRAP staining, and significance is compared with no flagellin treatment (0) in the suboptimal condition in (E) and (F). (**G**) Representative TRAP staining (original magnification ×200) of (F). Negative (−) and positive (+) controls consisted of untreated cells or cells treated with 20 ng/ml M CSF and RANKL. Values demonstrate mean ± SE. *$p < 0.05$.

by SF endogenous ligands can modulate monocyte homing through activation of PI3K/AKT1, JNK, and NF κB pathways.

TLR5 interconnects with TNF α in mediating monocyte chemotaxis

Ligation of TLR5 strongly induces production of TNF α in RA monocytes and macrophages (29); thus, we asked whether TLR5 expression can be affected by anti TNF α therapy. Interestingly, myeloid TLR5 expression was 2.5 fold higher in RA patients treated with DMARDs compared with those treated with anti TNF α agents (Fig. 2A), suggesting that these two pathways are cross regulated. Consistently, we demonstrate that TNF α and flagellin can synergistically induce myeloid cell migration (Fig. 2B), and hence blockade of both cascades can more potently suppress RA SF mediated monocyte chemotaxis compared with each pathway alone (Fig. 2C). These results indicate that in the RA joint, expression of TNF α triggered by myeloid TLR5 liga tion can further enhance TLR5 driven myeloid cell infiltration.

Myeloid TLR5 ligation activates RA osteoclast formation

Because TLR5 ligation contributes to RA joint myeloid cell che motaxis, we asked whether TLR5 activation could transform the recruited myeloid cells into mature osteoclasts. Experiments per formed established that PBMCs could be differentiated into fully

mature osteoclasts in the presence 20 ng/ml M CSF and RANKL, whereas the suboptimal conditions consisted of 10 ng/ml M CSF and RANKL in RA PBMCs (Fig. 2D). We next demonstrated that TLR5 ligation in NL and RA PBMCs could dose dependently contribute to osteoclast formation when cultured in suboptimal conditions (Fig. 2E G), suggesting that flagellin can promote transcription of essential osteoclastogenic factors.

We found that in RA PBMCs, flagellin treatment upregulates RANK, RANKL, and TNF α expression levels by 2 to 8 fold (Fig. 3A C). In contrast with our findings, others have shown that TLR5 ligation inhibits mouse bone marrow cell differentiation to mature osteoclast through IFN β induction (37); therefore, tran scription of IFN β was also assessed in our culture system. In terestingly, we document that there was an insignificant higher trend of IFN β expression in RA cells treated with flagellin compared with the PBS treatment (Fig. 3D).

Because RANKL is produced from RA T cells and fibroblasts, we asked whether TLR5 is expressed on T cells and whether these cells are critical for flagellin mediated osteoclastogenesis. Al though the cell surface TLR5 levels were not significantly higher, there was, however, a greater expression trend in RA compared with NL CD3^{+} T cells (Fig. 3E, 3F). We show that ligation of TLR5 could differentiate osteoclast precursor cells into fully mature osteoclasts in the absence of T cells when monocytes were
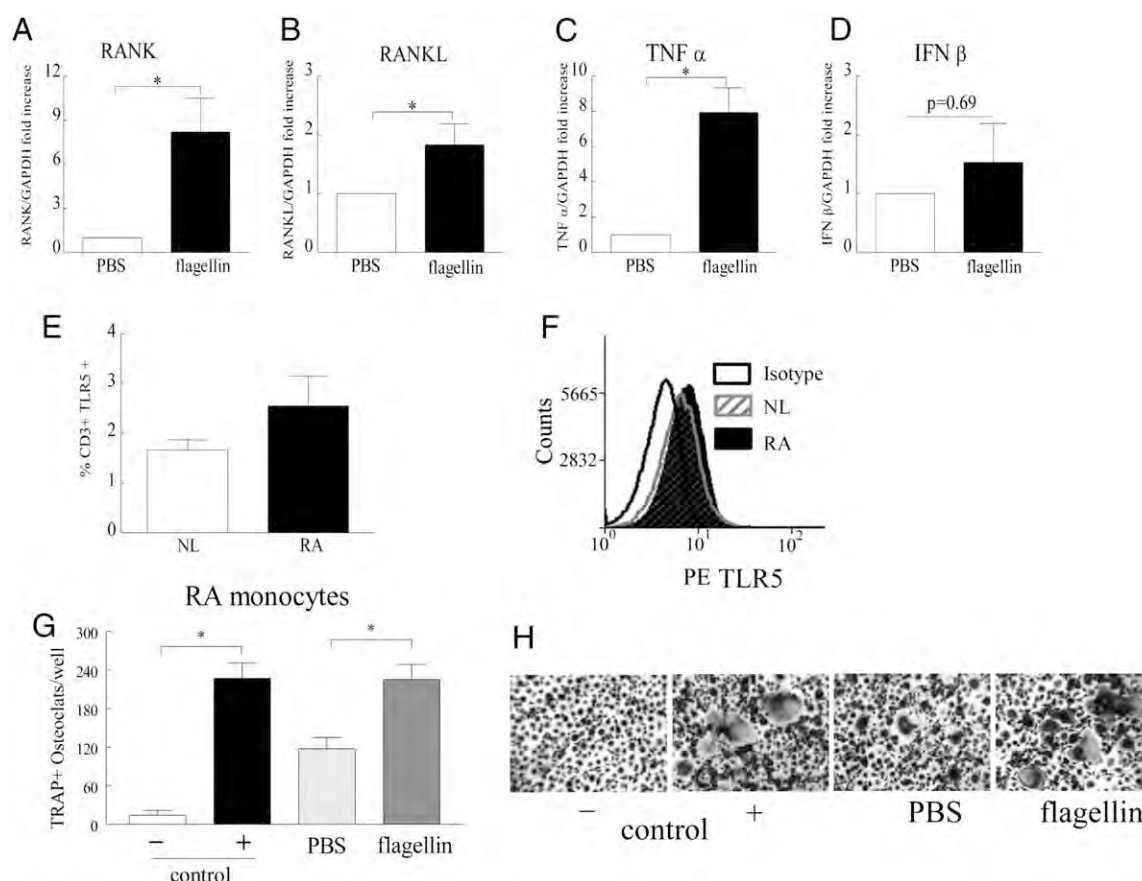

FIGURE 3. Ligation of TLR5 in RA PBMCs drives the transcription of pro osteoclastogenic factors, and TLR5 ligation promotes RA myeloid cells to form mature osteoclasts in suboptimal culture conditions. Using real time RT PCR, we quantified RANK (**A**), RANKL (**B**), TNF α (**C**), and IFN β (**D**) mRNA levels in RA osteoclast precursor cells that were cultured in suboptimal condition (10 ng/ml M CSF and RANKL) for 7 d before being treated with PBS or 10 ng/ml flagellin for 6 h in the absence of M CSF and RANKL (n 7). Results are shown as fold increase above the PBS group and are normalized to GAPDH. (**E**) NL and RA PBMCs were immunostained with FITC labeled anti CD3 Ab and PE conjugated anti TLR5 to determine the percentage of CD3^{+} and TLR5^{+} cells (n 3); (**F**) a representative flow cytometry histogram of (E). (**G**) Negatively selected RA monocytes were cultured in the suboptimal condition and were either untreated (PBS) or treated with 10 ng/ml flagellin before TRAP staining. (**H**) Representative TRAP staining (original magni fication ×200) of (G). Negative and positive control consisted of untreated cells or cells treated with 20 ng/ml M CSF and RANKL. Values demonstrate mean ± SE. *$p < 0.05$.

cultured in a suboptimal condition (Fig. 3G, 3H). Taken together, these results suggest that monocytes are the effector cells in TLR5 mediated osteoclastogenesis, and flagellin can facilitate osteoclast formation by increasing RANK expression and allow ing the cells to be more responsive to RANKL binding, resulting in less RANKL being required for this process. In addition, it is possible that the pro osteoclastogenic factors counterbalance the inhibitory effect of IFN β in part because flagellin induced TNF α transcription can potentiate RANK/RANKL cascade whereas suppressing the IFN β transcription (38).

TLR5 links with TNF α in enhancing joint osteoclastogenesis

Next, experiments were performed to document whether TNF α is capable of potentiating TLR5 mediated osteoclast formation. We show that TLR5 mediated osteoclastogenesis is in part due to TNF α produced from RA myeloid cells (Fig. 4A, 4B). However, because osteoclast differentiation driven by TLR5 and TNF α is dose dependent, supplementing the flagellin treated cells with exogenous TNF α further increased the number of TRAP+ osteoclasts by 2 fold (Fig. 4C, 4D). Confirming this notion, we show that in RA SF, TLR5 endogenous ligand(s) connect with TNF α in promoting osteoclastogenesis, because inhibition of both pathways can suppress RA SF mediated osteoclast formation

more potently compared with each pathway alone (Fig. 4E). Our results indicate that TLR5 and TNF α pathways are linked in fostering RA myeloid cell recruitment and osteoclast differentia tion.

Flagellin fosters osteoclastogenesis in mouse PB and bone marrow cells via TNF α, which is distinct from IFN β pathway

In contrast with previous findings (37), we document that ligation of TLR5 plays a critical role in osteoclastogenesis as determined in vitro in RA PB myeloid cells and in vivo in acute and chronic models of experimental arthritis. Therefore, experiments were performed for the first time, to our knowledge, in mouse PB myeloid cells, as well as in bone marrow cells, to address the data discrepancy. We show that flagellin activation transforms nega tively selected mouse PB monocytes cultured in suboptimal con ditions into multinuclei mature osteoclasts (Fig. 5A, 5B). Because of limited access to mouse PB monocytes (blood from 20 mice was used to obtain adequate mouse PB myeloid cells for 4 wells/ conditions), mouse bone marrow cells were used to determine the mechanism by which flagellin ligation to TLR5 promotes osteo clastogenesis. Consistent with our results in RA cells, we dem onstrate that blockade of TLR5 or TNF α in mouse bone marrow osteoclast precursor cells significantly reduces flagellin induced
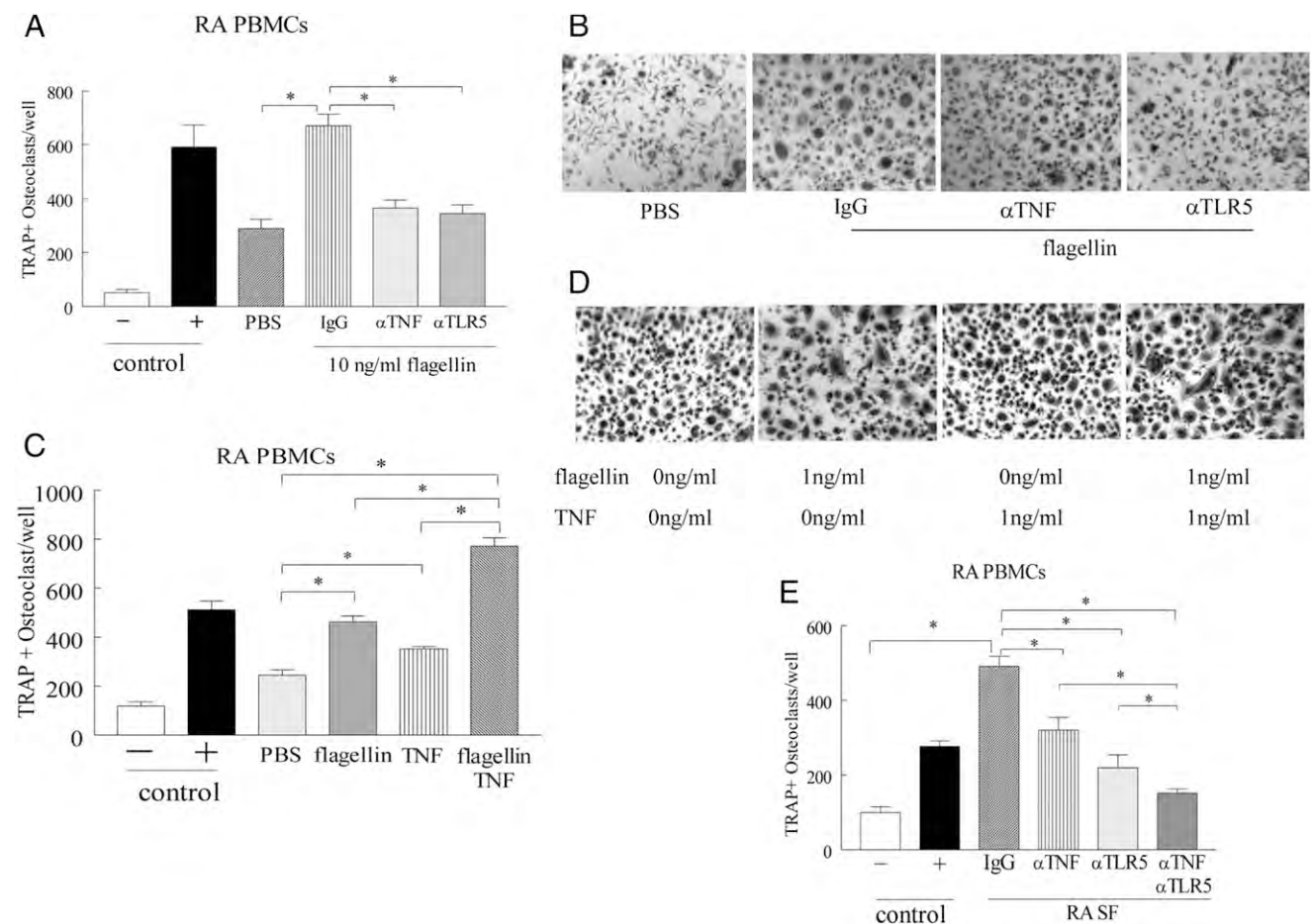

FIGURE 4. In RA joint, TLR5 and TNF α mediated osteoclastogenesis is interconnected. (**A**) RA PBMCs cultured in suboptimal conditions (10 μg/ml M CSF and RANKL) were untreated or pretreated with 10 μg/ml IgG, anti TNF α, and anti TLR5 Abs before being stimulated with 10 ng/ml flagellin, followed by TRAP staining. (**B**) A representative image of TRAP+ cells (original magnification ×200) from (A). n 3. (**C**) RA PBMCs were cultured in suboptimal condition and were either untreated or treated with 1 ng/ml flagellin, 1 ng/ml TNF α, or both before TRAP staining. (**D**) A representative image of TRAP staining (original magnification ×200) from (C). n 3. (**E**) RA PBMCs cultured in suboptimal condition were immunoneutralized by 10 μg/ml IgG control or anti TLR5 Abs, and cells were then incubated with 2% RA SF plus IgG or 2% RA SF plus anti TNF α (10 μg/ml) subsequent to TRAP staining (n 4). Negative and positive control consisted of untreated cells or cells treated with 20 ng/ml M CSF and RANKL. Values demonstrate mean ± SEM. *p < 0.05.
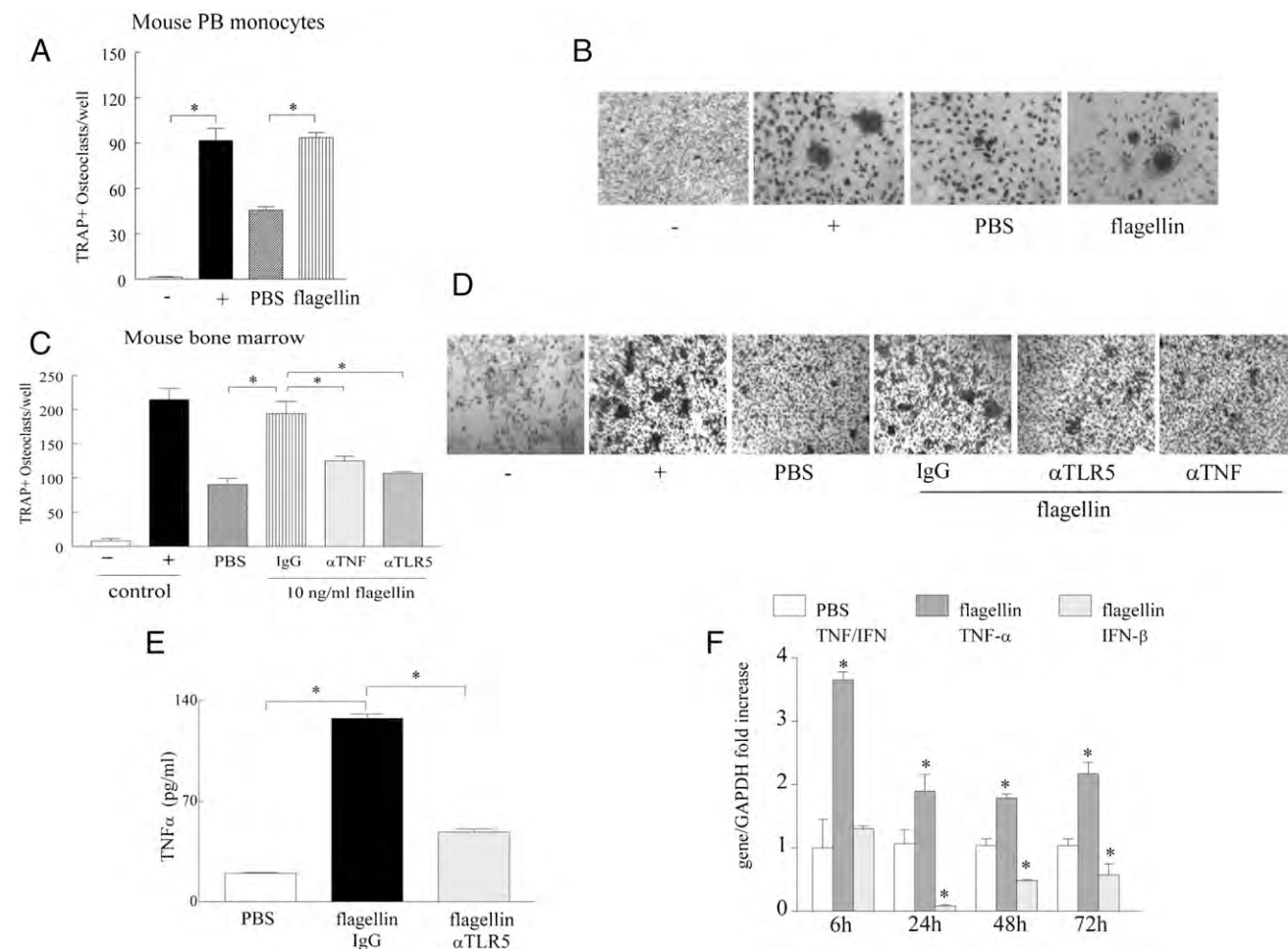
FIGURE 5. Flagellin strongly drives differentiation of murine PB monocytes and bone marrow cells to mature osteoclasts through TNF α activation. (**A**) Mouse PB monocytes were negatively selected and cultured in 10% α MEM, 20 ng/ml mouse M CSF, and RANKL. Mouse PB monocytes were either untreated (PBS) or treated with flagellin (10 ng/ml) for 14 21 d before TRAP staining. Mouse PB monocytes cultured in 10% α MEM alone were considered as negative control, and cells cultured in presence of 40 ng/ml M CSF and RANKL served as the positive control. (**B**) A representative image of TRAP+ cells (original magnification ×400) from (A) (*n* 3). (**C**) Mouse bone marrow cells cultured for 4 d in 10% α MEM, 10 ng/ml mouse M CSF plus 25 ng/ml mouse RANKL were untreated or stimulated with flagellin (10 ng/ml) plus IgG (10 μg/ml), flagellin (10 ng/ml) plus anti TLR5 (10 μg/ml), or flagellin (10 ng/ml) plus anti TNF α (10 μg/ml) for 3 additional days before TRAP staining (*n* 3). Mouse bone marrow cells cultured in 10% α MEM alone served as negative control, and cells supplemented with mouse 10 ng/ml M CSF and 100 ng/ml RANKL served as positive control. (**D**) A representative image of TRAP+ cells (original magnification ×200) from (C). (**E**) Mouse TNF α protein concentration was determined by ELISA in day 4 mouse bone marrow precursor cells cultured in 10% α MEM, 10 ng/ml mouse M CSF plus 25 ng/ml mouse RANKL either untreated (PBS) or treated with flagellin (100 ng/ml) plus IgG (10 μg/ml) versus flagellin (100 ng/ml) plus anti TLR5 (10 μg/ml) for 24 h (*n* 5). (**F**) TNF α and IFN β real time RT PCR was performed on mouse bone marrow cells from day 4 cultured in 10% α MEM, 10 ng/ml mouse M CSF plus 25 ng/ml mouse RANKL, and treated with or without flagellin (100 ng/ml) for 6, 24, 48, and 72 h (*n* 3). Values demonstrate mean ± SEM. *$p < 0.05$.

osteoclast maturation, in part, by inhibiting TNF α production (Fig. 5C E). In mouse bone marrow osteoclast precursor cells, flagellin treatment elevates TNF α transcription by 4 fold at 6 h, and these levels remain constantly 2 fold higher from 24 to 72 h of stimulation, whereas IFN β mRNA levels are not significantly accentuated at 6 h stimulation and are further reduced after 24 72 h of flagellin activation (Fig. 5F). These results suggest that similar to RA myeloid cells, ligation of murine TLR5 cells by flagellin can strongly promote osteoclast formation, in part, through production of TNF α and dysregulation of IFN β cascade.

Postonset treatment of CIA with flagellin contributes to elevated joint myeloid cell infiltration and osteoclastogenesis

We next asked whether acute and/or chronic arthritis driven by TLR5 ligation is due to homing and differentiation of myeloid cells into mature osteoclasts. We document that when CIA mice were therapeutically treated with TLR5 agonist, joint swelling was

markedly greater in mice that received flagellin treatment compared with PBS control (Fig. 6A, 6B). Similar to our in vitro studies, we found that postonset treatment with flagellin could strongly facilitate myeloid cell recruitment into the CIA ankle joints (Fig. 6C, 6D). Further, joint TNF α production levels were 5.5 fold higher, whereas transcription of IFN β was unchanged in CIA mice that received postonset treatment of flagellin compared with the control group (Fig. 6E, 6F). We also show that the number of TRAP+ cells (Fig. 7A, 7B) and the concentration of bone erosion markers CTR, CTSK, and RANKL (Fig. 7C) were lower in the control ankles compared with the flagellin treated CIA joints. These results indicate that the elevated joint myeloid cell migration and their differentiation to osteoclasts may be caused by TLR5 ligation, as well as TNF α production, or it is possible that both mechanisms of action contribute to the detected observations.

Because CIA ankle swelling and bone erosion were exacerbated by flagellin postonset treatment, we next asked whether local in
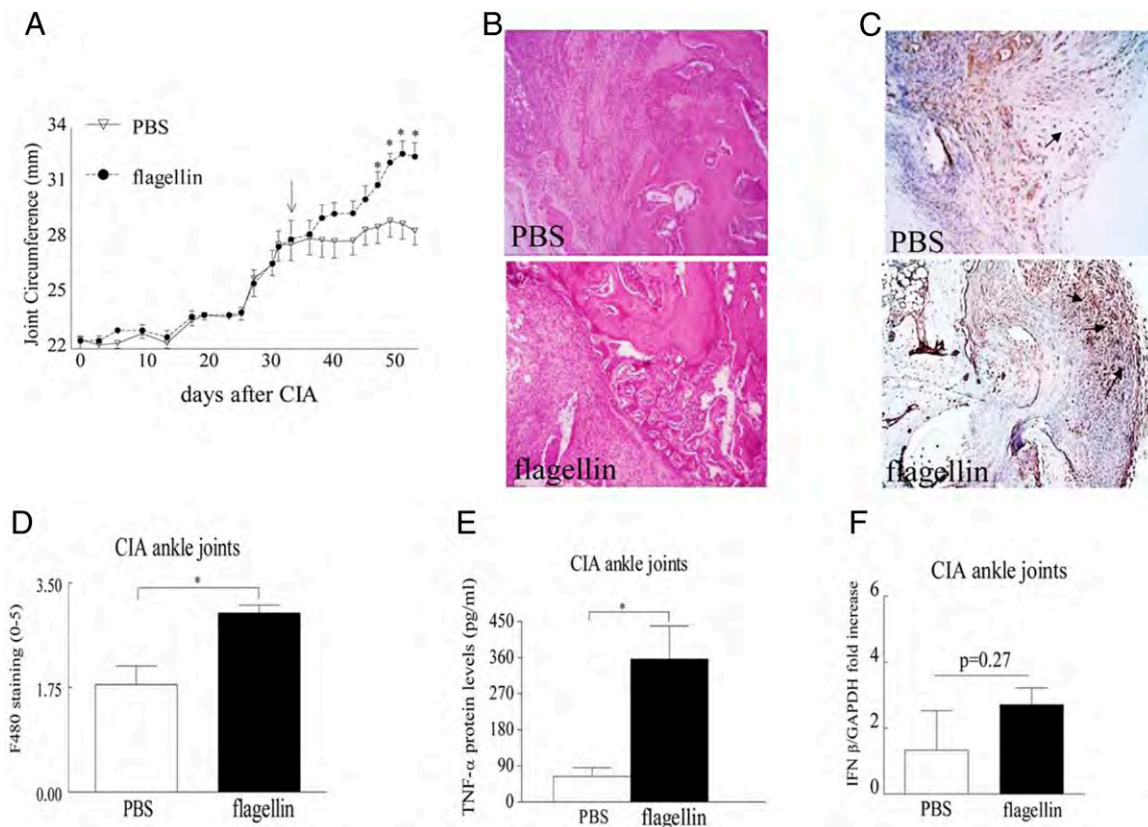
FIGURE 6. Flagellin postonset treatment in CIA contributes to joint inflammation, elevated joint myeloid cell trafficking, and TNF α production. (**A**) Changes in joint circumference were determined in CIA mice that were treated i.p. with PBS or flagellin (20 μg) on day 33 (n   10). (**B**) Ankles were harvested on day 57 from CIA mice treated with PBS or flagellin and were H&E stained (original magnification ×200; n   7). (**C**) STs from CIA mice treated with PBS or flagellin were harvested on day 57 and were immunostained with anti F480 Ab (original magnification ×200). Arrows demonstrate F480+ cells. (**D**) Macrophage staining was quantified on a 0 5 scale (n   7). (**E**) TNF α protein levels (pg/ml) were quantified by ELISA in ankle homogenates from CIA mice treated with PBS or flagellin (n   7). (**F**) Transcription of IFN β was determined by real time RT PCR in CIA ankles that had received postonset treatment of flagellin or control, and the data are shown as fold increase above PBS group and are normalized to GAPDH (n   5).Values are mean ± SE. *$p < 0.05$.

jection of flagellin alone could drive joint inflammation and osteoclastogenesis. We demonstrate that ectopic TLR5 ligation elevates ankle circumference from days 0 to 2, and subsequently joint inflammation plateaus until day 8; however, swelling remains consistently higher than the PBS group (Fig. 7D, 7E). Corrobo rating with the CIA data, i.a. injection with flagellin resulted in 10 fold greater mature osteoclasts compared with the control group (Fig. 7F, 7G). Hence, consistent with our in vitro studies, data generated in acute and chronic animal models suggest that flagellin ligation to joint TLR5 contributes directly, as well as indirectly, to myeloid cell infiltration and osteoclast maturation.

Blockade of TLR5 function impairs CIA joint monocyte infiltration and osteoclast formation

To document the critical role of TLR5 in RA pathogenesis, we systemically treated CIA mice with IgG or anti TLR5 Ab. Results from these experiments demonstrate that CIA mice treated with anti TLR5 Ab have markedly reduced joint swelling starting on day 44 until day 48 compared with the IgG control group (Fig. 8A C). When the underlying mechanism of function was examined, we found that blockade of TLR5 inhibited F480CD80+iNOS+ M1 macrophage differentiation in CIA ankle joints (Fig. 8D, 8E) which resulted in significantly lower production of joint IL 6 and CCL2 protein levels compared with the IgG control group (Fig. 8F). In addition, we show that joint myeloid cell migration and their remodeling to mature osteoclasts were compromised in the anti TLR5 Ab group compared with the control group

(Fig. 8G, 8H). Because blockade of TLR5 function ameliorates joint myeloid cell trafficking and their transformation into M1 macrophages and/or fully mature osteoclasts, these results suggest that TLR5 can be used as a target for RA therapy.

Discussion

In this study, we demonstrate for the first time, to our knowledge, that myeloid TLR5 ligation to endogenous ligands expressed in the RA joint strongly promotes monocyte trafficking and osteo clast formation. We document that TLR5 and TNF α pathways are cross regulated, because ligation of TLR5 in RA and mouse osteoclast precursor cells activates TNF α production, and anti TNF α therapy markedly reduces RA myeloid TLR5 expression. Flagellin treatment in mouse PB and bone marrow cells, as well as in CIA ankle joints, reveals that joint TLR5 ligation contributes to elevated osteoclast maturation through a TNF α dependent and IFN β independent mechanism. Finally, alleviation of joint in flammation and bone destruction by anti TLR5 Ab therapy in CIA further establishes TLR5 as a novel RA therapeutic target.

Our initial observation that TLR5 expression is elevated in RA myeloid cells and has a close correlation with disease activity and myeloid TNF α concentration (29) triggered our interest in unraveling how ligation of myeloid TLR5 impacts RA patho genesis. Because increase in the number of joint myeloid cells can be caused by increased chemotaxis, reduced efflux, or cell death, we examined the role of TLR5 in monocyte chemotaxis. We demonstrate that RA SF TLR5 endogenous ligand(s) participate in
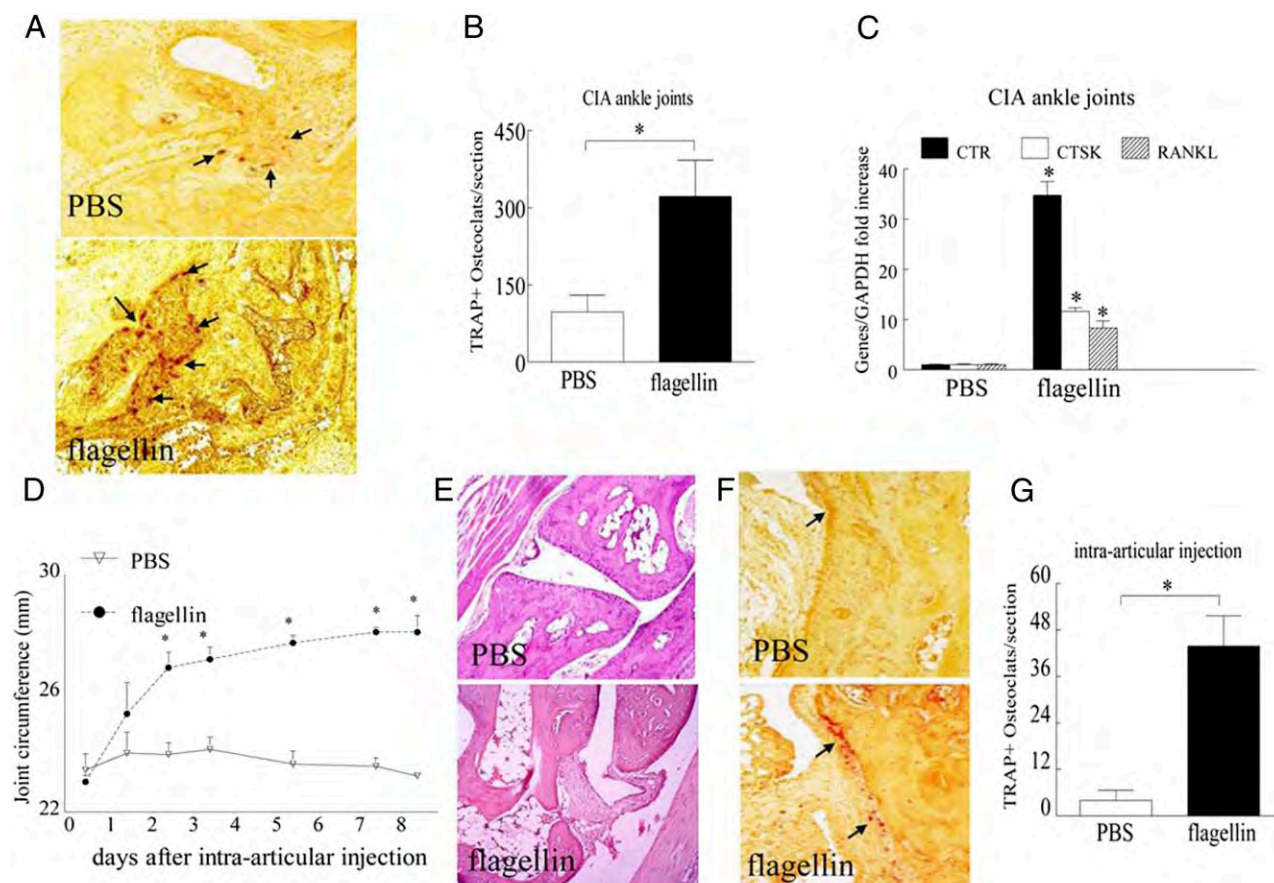
FIGURE 7. TLR5 ligation facilitates osteoclast formation in vivo in RA animal models. (**A**) Ankles harvested from CIA mice treated with PBS or flagellin were TRAP stained (TRAP$^+$ cells are shown by arrows) (original magnification ×200), and (**B**) the number of TRAP$^+$ cells were counted per section (n 7). (**C**) Transcription of CTR, CTSK, and RANKL was determined by real time RT PCR in CIA ankles that had received postonset treatment of flagellin or control, and the data are shown as fold increase above PBS group and are normalized to GAPDH (n 5). (**D**) Changes in joint circumference in mice i.a. injected with PBS or 20 μg flagellin (n 5). Ankles harvested from local injection of PBS or flagellin on day 10 were H&E (**E**) and TRAP (**F**) stained (TRAP$^+$ cells are shown by arrows) (original magnification ×200), and (**G**) number of TRAP$^+$ cells were counted per section (n 5). Values are mean ± SE. *$p < 0.05$.

joint monocyte homing through activation of PI3K/AKT, JNK, and NF κB, which is distinct from the pathway used by clas sical monocyte chemoattractants such as CCL2/MCP 1, CCL5/ RANTES, CCL3/MIP 1α, fMLF, and IL 17 that use p38 cas cade (14, 39). We further demonstrate that TLR5 induced mono cyte migration is potentiated by TNF α, as blockade of both pathways can more efficiently reduce RA SF mediated myeloid cell recruitment. TNF α is shown to be a potent in vitro (13) and in vivo (40) monocyte chemoattractant that facilitates monocyte transendothelial migration, in part, via enhancing endothelial adhesion molecules ICAM 1 and CD44 (41). Consistently, RA responders to anti TNF α therapy have significantly reduced numbers of RA ST CD68$^+$ sublining macrophages (42). Others demonstrate that transendothelial monocyte trafficking mediated by TLR4 ligation is directly facilitated via induction of PECAM1 on endothelial cells (43) and indirectly through production of CCL2/MCP 1 from myeloid cells (44), and that anti TNF α treatment had no effect on this function (45). This suggests that monocyte trafficking driven by TLR4 is differentially modulated compared with TLR5, and consequently, this process is unasso ciated with the TNF pathway.

Our novel data demonstrate that in RA PBMCs, TLR5 ligation can potently facilitate osteoclast differentiation by activating transcription of RANK and TNF α from myeloid cells and

RANKL from T cells. Interestingly, upregulation of RANK on myeloid cells and further production of TNF α from osteoclast precursors is adequate for promoting TLR5 mediated osteoclast formation, and T cell presence is not required when suboptimal doses of RANKL are used. Previous studies demonstrate that TLR4 ligation can promote osteoclast formation in RANKL pre treated mouse bone marrow cells through a TNF α related cascade (21). However, when mouse preosteoclasts were simultaneously treated with LPS, RANKL, and M CSF, osteoclast maturation was suppressed by TLR4 ligation despite elevated TNF α production (21, 23). It was shown that the inhibitory effect of TLR4 ligation on osteoclastogenesis was due to downregulation of RANK, as well as elevated expression of osteoprotegerin or IFN γ (22, 24). Others have shown that similar to TLR4, ligation of TLR5 inhibits transformation of mouse bone marrow cells to differentiated osteoclasts through IFN β induction (37). In contrast with the previous studies (37), we document that in RA and murine cells, as well as in CIA ankles, flagellin treatment was unable to enhance IFN β transcription, and this may be, in part, because of the robust TNF α transcription, because earlier findings demonstrate that TNF α signaling can counterbalance type I (IFN β and other isoforms) and type II (IFN γ) IFN function (38, 46 48).

Much like our findings in RA (29), earlier studies demonstrate that TLR5 is elevated in cancer compared with NL lingual epi thelium and may be a novel predictive marker for tongue cancer
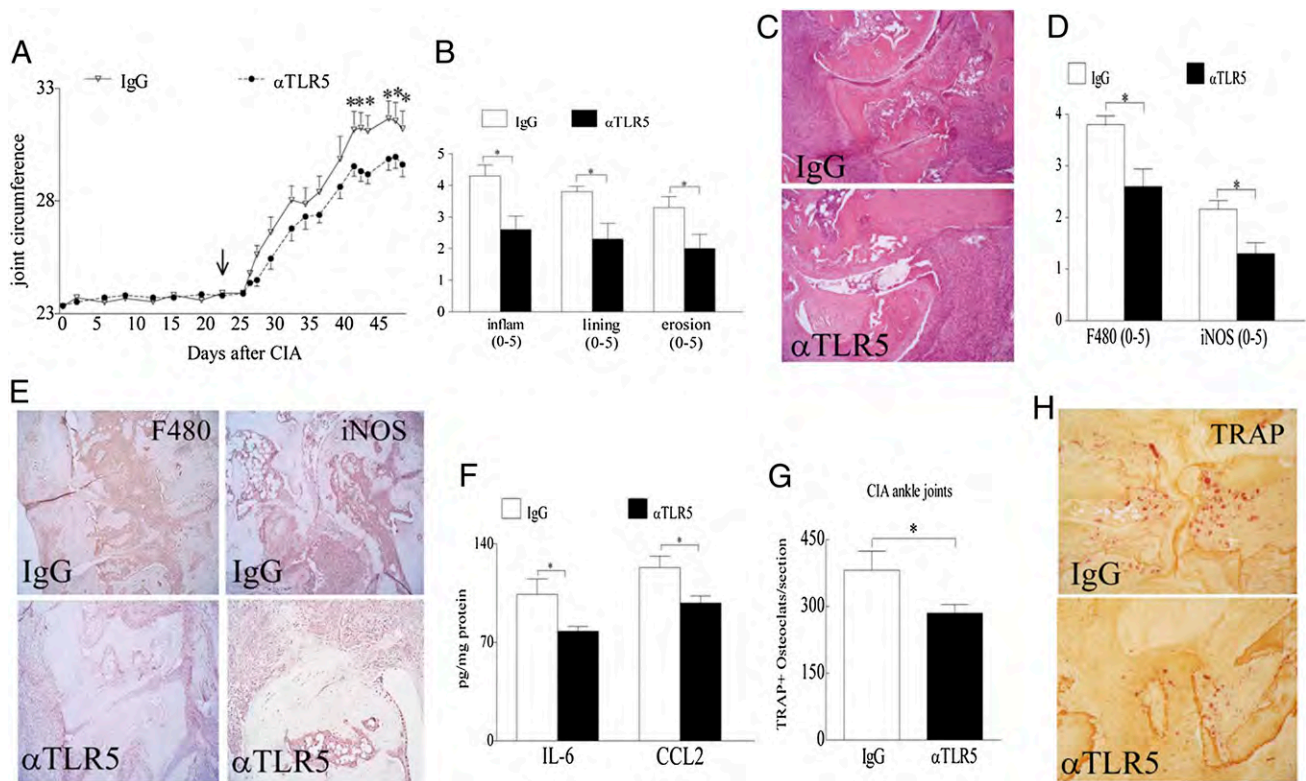
FIGURE 8. Anti TLR5 Ab treatment alleviates CIA joint swelling and bone resorption. (**A**) Changes in joint circumference were recorded for CIA mice that were treated i.p. with IgG or anti TLR5 Ab (100 μg/mouse) on days 23, 27, 30, 34, 37, 41, 44, and 48, and mice were sacrificed on day 49 after induction, *n* 6 mice (12 ankles). (**B**) Effect of anti TLR5 Ab treatment on inflammation, lining thickness, and bone erosion was scored on a 0 5 scale (*n* 6). (**C**) Representative ankle H&E staining (original magnification ×200) of (B). (**D**) STs from CIA mice treated with IgG or anti TLR5 Ab were harvested on day 49 and immunostained with anti F480 (1:100 dilution) or iNOS Abs (1:200 dilution) (original magnification × 200). Joint myeloid cells and iNOS$^+$ M1 macrophage staining were quantified on a 0 5 scale (*n* 6). (**E**) Representative F480 and iNOS immunostaining (original magnification ×200) of (D). (**F**) Changes in IL 6 and CCL2 protein levels in ankle homogenates from CIA mice treated with IgG control or anti TLR5 Ab were determined by ELISA (*n* 6). (**G**) Number of TRAP$^+$ cells were counted per section in CIA mice treated with IgG or anti TLR5 Ab (*n* 6). (**H**) Representative ankle TRAP staining (original magnification ×200) of (G). Values are mean ± SE. *$p < 0.05$.

recurrence (49). Notably, although flagellin can strongly facilitate migration and invasion of the salivary gland adenocarcinoma cell line, LPS and Pam3CSK4 did not have any effect on these func tions (50), suggesting that TLR5 shows some unique character istics that do not overlap with other TLRs both in cancer and RA.

Despite extensive studies, controversial results were obtained when the effect of TLR4 ligation was examined on osteoclasto genesis of mouse bone marrow cells because the outcome was heavily dependent on the cell treatment conditions (21 23). Therefore, in this study, the pathogenic role of TLR5 was assessed in RA and mouse cells, as well as in experimental arthritis models. Unlike results generated in mouse bone marrow studies, ligation of TLR2 and TLR4 in RA ST fibroblasts cocultured with myeloid cells could strongly promote osteoclast formation through induc tion of IL 1β and RANKL (51). In contrast with TLR2 and TLR4 mediated osteoclastogenesis, which is driven by IL 1β (51), our data demonstrate a strong connection between TLR5 and TNF α in joint bone erosion.

Notably, it was demonstrated by two independent groups that ligation of TNFR1 and TNFR2 by TNF α could strongly drive osteoclast differentiation, and this process was unaffected by blockade of RANKL via osteoprotegerin (10, 11). Confirming this finding, others show that macrophages from RA SF can differ entiate to fully mature osteoclasts in the presence of M CSF with RANKL or TNF α/IL 1β, suggesting that factors such as TNF α and IL β can substitute RANKL's function in the RA joint (12). The same group found that osteoclast formation was more pro

nounced in RA SF that contained higher TNF α levels (52). We document that transcription of RA SF TNF α is modulated by SF TLR5 endogenous ligands (29), and that TLR5 interconnects with TNF α in enhancing joint osteoclastogenesis. Therefore, these observations indicate that SF with most abundant TNF α may be a result of elevated TLR5 endogenous ligands that contribute to markedly enhanced bone degradation.

Interestingly, a number of endogenous ligands have been identified that can activate TLR4 function (including fibrinogen, surfactant protein A, fibronectin extra domain A, heparan sulfate, soluble hyaluronan, and defensin 2), whereas other endogenous ligands can trigger both TLR2 and TLR4 pathways (such as heat shock proteins [HSPs; HSP60, 70, and 96] and HMGB1) (53, 54). Most recently, RNA extracted from RA SF was shown to be a TLR7 endogenous ligand that could dose dependently modulate RA SF TNF α transcription (55). Earlier studies demonstrate that stimulation with HSP70 can further potentiate flagellin mediated NF κB luciferase activity in TLR5 transfected HEK 293T reporter cells, but because this effect was not detected with HSP70 treat ment alone, these results suggest that HSP70 may operate as a chaperone protein for TLR5 endogenous ligands (56). There is still a possibility that other HSPs can serve as a potential TLR5 endogenous ligand in the RA joint; therefore, experiments are currently being conducted to identify these TLR5 endogenous ligands in RA SFs and STs.

Although gain of TLR5 function promotes myeloid cell re cruitment and osteoclast formation partly through potentiating the

joint TNF α production, loss of TLR5 function reverses both mechanism of function, in part, by suppressing the proin flammatory M1 macrophage differentiation process in CIA ankle joints. In contrast, joint inflammation and bone erosion mediated by local injection of TNF α was unaffected in TLR2 and TLR4 deficient mice, suggesting that TNF α driven joint pathology is independent of TLR2 and TLR4 function (26). In contrast, se verity of IL 1 induced bone erosion and cartilage destruction was markedly suppressed in TLR4 $^{/}$ mice. Similarly, TLR4 induced calvarial bone resorption was significantly reduced in IL 1R $^{/}$ compared with wild type mice (28). These results suggest that although TLR5 mediated myeloid cell trafficking and differenti ation to osteoclasts are linked to TNF α pathway, TLR4 induced monocyte infiltration is unrelated to TNF α, whereas its bone erosion is dependent on IL 1β.

In summary, we show that SF TLR5 endogenous ligands par ticipate in recruiting circulating myeloid cells into the joint and further facilitating their differentiation into mature osteoclasts, and both functions are interconnected to joint TNF α cascade. Thus, the connection between TLR5 and TNF cascade and the pre clinical evidence obtained from TLR5 loss of function highlight the importance of this receptor in RA disease.

Disclosures

The authors have no financial conflicts of interest.

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